

**The Role of Some Putative Mediators of Peripheral
Nociceptor Activation in Adjuvant–Induced
Experimental Arthritis**

By

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These things are sent to try us, forge our characters, and make us strong.

- Jack Gauldie
(Most likely stolen from a bathroom wall)

Declaration

I declare that this thesis was composed entirely by myself, and the work on which it is based is my own with the following exceptions noted below and acknowledged in the text:

Histological samples were processed, paraffin embedded, sectioned and stained by Mr. Barry Reed (GlaxoSmithKline, Ware, UK)

Dr. Christopher Clarke (GlaxoSmithKline, Ware, UK) graded the pathology of mouse knee joint histological sections

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Abstract

The development of better analgesic drugs for the treatment of chronic inflammatory conditions such as rheumatoid arthritis (RA) would be greatly facilitated by extending knowledge of the underlying mechanisms involved in generating pain in diseased tissue and developing new models for testing candidate compounds. In this thesis behavioural and electrophysiological techniques in both the rat and the mouse were used to determine the effect of a number of mediators and/or pharmacological receptors reported to be involved in chronic inflammation and nociception. The specific aims of the thesis centred on testing the following hypotheses: a) The neuropeptide somatostatin inhibits sensory nerve function in both normal and arthritic knee joints, b) the endocannabinoid anandamide activates peripheral nociceptors via its reported action at the vanilloid receptor (VR₁), c) chronic unilateral inflammation of the knee joint can be induced in mice using Freund's Complete Adjuvant (FCA), and d) the purinoceptor P2X₇ plays a role in the induction of inflammation and hyperalgesia associated with experimental arthritis in mice. An additional aim of the thesis was *in vivo* neural recording from nociceptors innervating the mouse knee joint with a view to examining transgenic mice in future studies.

Somatostatin or its long lasting analogue octreotide, and anandamide were administered by close intra-arterial injection to both normal and FCA-induced arthritic rat knee joints and their ability to inhibit and/or excite articular nociceptors was recorded under pentobarbitone anaesthesia. Furthermore, the ability of these agents to modulate afferent discharge caused by known algogens (capsaicin (VR₁ receptor), bradykinin (B₁/B₂ receptor), and α,β -methylene adenosine 5' triphosphate

($\alpha\beta$ meATP; P2X₁/P2X₃ receptor)) was also investigated. Neither somatostatin nor octreotide directly activated peripheral nociceptors in normal or arthritic rat knee joints. Furthermore, neither compound inhibited either basal firing or discharge evoked by capsaicin, bradykinin or $\alpha\beta$ meATP. Anandamide caused a rapid onset, short duration increase in afferent discharge in both normal and arthritic rat knee joints. This was via the VR₁ receptor because the response was blocked by the VR₁ antagonist capsazepine.

Chronic unilateral inflammation induced by intra-articular injection of FCA was characterised in two mouse strains, DBA/1 and C57 Black 6 (C57BL6). The developed model was used to examine the role of the purinoceptor P2X₇ (ATP nucleotide receptor) in the induction of experimental arthritis by using a transgenic mouse line lacking the P2X₇ receptor. To enable the use of transgenic mice for studying chemically or mechanically evoked responses in peripheral nociception, a technique was developed for recording afferent discharge from the saphenous nerve of the anaesthetised mouse *in vivo*.

Behavioural studies in mice showed that repeated intra-articular injections of FCA produced a persistent and local inflammation in the knee joint that was associated with hyperalgesia to mildly noxious pressure. Histology of the joints showed changes characteristic of a mild, unilateral arthritis present in all treated animals. The inflammation induced was consistent between animals and between both DBA/1 and C57BL6 strains. Validation of the model was shown by the effective attenuation of the induced inflammation by treatment with prednisolone and indomethacin. The P2X₇ receptor appeared not to play a role in the inflammation or associated hyperalgesia produced in the mouse model of arthritis, as there was no

difference between knock out mice and wild-type controls in the arthritis and hyperalgesia induced by FCA. Finally, for the first time it has been possible to record chemically and mechanically evoked responses from nociceptors innervating normal and arthritic mouse knee joints under urethane anaesthesia.

In summary, in relation to the hypotheses being tested, the results showed that: a) the reported anti-nociceptive effect of somatostatin is not mediated by action on peripheral nociceptors or the inhibition of tested algogens, b) anandamide is able to directly activate sensory afferents via VR₁ receptors, c) chronic, unilateral arthritis can be induced in mice by repeated intra-articular injections of FCA, and d) P2X₇ purinoceptors do not play a role in the induction of inflammation and hyperalgesia associated with the FCA model of unilateral arthritis. Innovation in this thesis included a novel model of murine unilateral arthritis and the development of a new technique for direct measurement of evoked discharge from peripheral nociceptors innervating the mouse knee joint. These advances in knowledge provide information relevant to understanding inflammatory joint disease and for future drug development.

Abbreviations

%	percent
α	alpha
ANOVA	analysis of variance
ATP	adenosine 5' triphosphate
β	beta
$^{\circ}\text{C}$	degrees centigrade
Ca^{2+}	calcium ion
CNS	central nervous system
COX-1	cyclooxygenase 1
COX-2	cyclooxygenase 2
DNA	deoxyribonucleic acid
DRG	dorsal root ganglia
EC_{50}	the concentration producing 50% of the maximum response
FCA	Freund's complete adjuvant
G	gram
GPCR	G-protein coupled receptor
G-protein	guanine nucleotide binding regulatory protein
H	hour
H&E	haematoxylin/eosin
H^{+}	hydrogen ion (proton)
HLP	Heavy liquid paraffin
i.a.	intra-arterial
i.art.	intra-articular
i.p.	intra-peritoneal
K	kilo (10^3)
K^{+}	potassium ion
μ	micro (10^{-6})
M	metre
M	moles/litre

m (prefix)	milli (10^{-3})
MAN	medial articular nerve
Min	minute
MmHg	millimeters of mercury
Mol	mole
n	number
n (prefix)	nano (10^{-9})
Na⁺	sodium ion
NSAID	non-steroidal anti-inflammatory
OD	outer diameter
P	statistical probability
PBS	phosphate buffered saline
PG	prostaglandin
PLC	phospholipase C
PNS	peripheral nervous system
S	second
SCW	Streptococcal cell walls
SEM	standard error of the mean
TG	trigeminal ganglion
TX	thromboxane
v/v	volume per volume
w/v	weight per volume

Most abbreviations, other than commonly used expressions, are also defined at the first point of occurrence in the text.

Associated Publications

GAULDIE, S.D., MCQUEEN, D.S., PERTWEE, R. & CHESSELL, I.P. (2001). Anandamide activates peripheral nociceptors in normal and arthritic rat knee joints. *British Journal of Pharmacology*, **132**, 617-21.

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Table of Contents

Declaration.....	i
Acknowledgements	ii
Abbreviations	vi
Associated Publications	viii
1. General Introduction	16
Pain and nociception.....	17
Discovery and classification of nociceptors	18
Excitation of nociceptors	20
Mechanical excitation.....	20
Thermal excitation	21
Chemical excitation	22
Sensitisation of nociceptors	22
Arthritis.....	24
Knee joint articular afferents	26
Anatomy of articular afferents.....	27
Inflammatory mediators in nociception.....	32
Directly acting algogenic mediators	32
Indirectly acting algogenic mediators.....	38
Aims of the studies	41
2. Methods.....	43
2.1 Models of experimental arthritis.....	44
Rat model of unilateral arthritis.....	44
Induction of unilateral arthritis	44
Assessment of arthritis.....	45

Murine model of unilateral arthritis.....	47
2.2 In vivo recording from afferent nerves.....	47
Recording from afferent nerves in the rat knee joint.....	47
Anaesthesia and surgical procedures	47
Dissection of the medial articular nerve	48
Recording from the medial articular nerve	48
Recording from afferent nerves in the mouse knee joint.....	49
Identification of fibres	49
Mechanosensitivity	50
Conduction velocity.....	50
Spike duration.....	51
Chemosensitivity	51
Drug administration	51
Data sorting.....	52
Data analysis.....	52
Statistical analysis.....	53
Tables and Figures.....	55
3. Characterisation of nociceptors in normal and arthritic rat knee joints	58
3.1 Introduction.....	59
3.2 Methods	61
Afferent nerve recording in vivo	61
3.3 Results.....	62
Afferent nerve recordings <i>in vivo</i>	62

Characterisation of afferent nerve fibres	62
Spontaneous discharge in nociceptors innervating normal and arthritic rat knee joints.....	63
Evoked responses to known algogens capsaicin, bradykinin and $\alpha\beta$ meATP	64
3.4 Discussion.....	67
Tables and Figures.....	75
4. Effect of somatostatin on knee joint nociceptors	83
4.1 Introduction.....	84
4.2 Methods	88
Afferent nerve recording <i>in vivo</i>	88
4.3 Results.....	88
Characterisation of afferent nerve fibres	88
Neither octreotide nor somatostatin-14 excited peripheral nociceptors	89
Effect of octreotide on spontaneous afferent discharge from normal and arthritic knee joints.....	90
Effect of octreotide on responses from knee joint afferents evoked by capsaicin, bradykinin and $\alpha\beta$ meATP	91
4.4 Discussion.....	93
Tables and Figures.....	97
5. Effect of anandamide on knee joint nociceptors.....	101
5.1 Introduction.....	102
Anandamide as a neuromodulator in nociception	103
5.2 Methods	105
Afferent Nerve Recording <i>In Vivo</i>	105

5.3 Results.....	105
Characterisation of afferent nerve fibres	105
Anandamide-evoked activation of knee joint afferents	106
Proportion of afferents activated by anandamide	106
Dose-related increase in the nociceptor action potential discharge evoked by anandamide	107
Features of the anandamide-evoked response	107
Effect of anandamide on excitatory responses evoked by ATP, bradykinin and capsaicin	109
Comparison of the capsaicin and anandamide-evoked responses in normal knee joints	110
Response to anandamide is abolished by the VR ₁ antagonist, capsazepine	111
5.4 Discussion.....	112
Tables and Figures.....	115
6. FCA-induced unilateral arthritis in the mouse knee joint.....	125
6.1 Introduction.....	126
Murine models of arthritis	127
Transgenic mouse strains and arthritis	128
Aims of the study.....	130
6.2 Methods	131
Induction of unilateral arthritis	131
Assessment of arthritis.....	131
Body Weight.....	132
Inflammation.....	132

Hyperalgesia/weight bearing allodynia	132
Drug treatment	133
Histopathology	133
Electrophysiological recording from the saphenous nerve.....	135
6.3 Results.....	136
Induction of unilateral arthritis in mice	136
Sub-cutaneous injection of FCA around the mouse ankle joint	136
Sub-cutaneous injection of FCA around the mouse knee joint	137
Antigen induced arthritis with methylated bovine serum albumin.....	138
Unilateral arthritis with repeated injections of FCA in DBA/1 mice	139
Unilateral arthritis with repeated injections of FCA in C57BL6 mice	143
Comparison of unilateral arthritis development in different strains of mice....	148
Knee joint diameter.....	148
Hyperalgesia/allodynia	148
Histopathology.....	149
Effect of the steroid prednisolone on established FCA-induced unilateral arthritis DBA/1 mice.....	150
Knee joint diameter.....	150
Body weight.....	151
Hyperalgesia/allodynia	152
Histopathology.....	153
Effect of NSAID indomethacin on established FCA-induced unilateral arthritis in C57BL6 mice.....	154
Knee joint diameter.....	155

Body weight.....	156
Hyperalgesia/allodynia	156
Histopathology.....	158
Induction of FCA unilateral arthritis in P2X ₇ knock out mice	159
Background.....	159
Knee joint diameter.....	160
Body weight.....	161
Hyperalgesia/allodynia	162
Histopathology.....	163
Recording from the saphenous nerve in normal and arthritic knee joints of C57BL6 mice.....	164
Basal discharge of nociceptors innervating normal and arthritic knee joints	165
Dose-related increase in the action potential discharge evoked by capsaicin and bradykinin	166
Features of the capsaicin and bradykinin evoked responses	166
6.4 Discussion.....	167
Tables and Figures.....	180
7. General Discussion and Future Directions.....	227
7.1 Properties of nociceptors innervating normal and arthritic rat knee joint	228
Future directions	231
7.2 Modulation of nociceptors by inflammatory mediators	232
Future directions	234

7.3 Murine unilateral arthritis and electrophysiological recording from afferents innervating the mouse knee joint.....	235
FCA-induced unilateral arthritis in the mouse knee joint.....	235
Future directions	236
Electrophysiological recording from mouse knee joint afferents in vivo.....	237
Future directions	237
7.4 General conclusions.....	238
References	240
Index of Figures and Tables.....	261
Appendix I - Drugs and Solutions	266
Appendix II - Publications	268

1. General Introduction

Arthritic conditions, including rheumatoid and osteoarthritis (RA and OA respectively) affect a large number of people worldwide, with over 8 million people in the UK suffering from arthritis, including up to 3 million who are severely disabled by the condition (Arthritis-Research-Foundation, 2002). “Arthritis” simply means an inflammation of the joint and the primary clinical feature of the disease is chronic inflammation that is capable of affecting all joints in the body, but most commonly affects the toes, ankles, knees, shoulders, wrists, and knuckles (Kelley *et al.*, 1997). Inflammation within the joint causes the accumulation of inflammatory cells in the synovial space, the release of lysosomal enzymes resulting in bone and cartilage erosion, oedema and deformation, and bone formation that can fuse the joint together and impair normal mobility. The chief complaint of patients presenting with an arthritic condition is severe chronic pain. Current therapies for arthritis involve reducing the chronic inflammation in the hopes of decreasing the associated pain.

PAIN AND NOCICEPTION

One of the vital roles of the sensory (afferent) nervous system is to provide information about our external surroundings and the state of various internal tissues for the purpose of homeostasis. This includes information about things in our surroundings that have the potential to cause injury. The sensation of pain is emotive, and as such strongly reinforces our reaction to painful stimuli. This contributes to our ability to avoid the same or related stimuli when we come across them in the future, thereby preventing injury. Additionally, if an injury is sustained the perception of pain causes a reflex protective action that prevents further injury.

Pain, although an important consideration in conditions such as RA, especially for those who suffer from it, is merely the subjective perception of sensory input arising from a noxious stimulus.

DISCOVERY AND CLASSIFICATION OF NOCICEPTORS

Psychophysicists such as (Blix, 1884) and (von Frey, 1894; von Frey, 1922) provided the first evidence that pain was a sense distinct from touch and was mediated by specialised sensory organs. Von Frey was able to describe different sensitivities to stimuli such as touch, pain, warmth and cold at precise points on the skin. Further more he was able to show that at spots producing pain the area was exclusively innervated by unspecialised or “free” endings – so called because the afferents do not terminate at a specific sensory structure or endplate. These “free” endings were termed “noci-receptors” by Sherrington and it was proposed that they existed to detect external stimuli that was potentially harmful (Sherrington, 1906).

Later, work using electrophysiological recording techniques from sensory nerves innervating peripheral tissues, showed that there were several kinds of fibres responsible for the transmission of sensory information (Clarke *et al.*, 1935; Gasser & Erlanger, 1927). The classification of these afferent fibres divides the afferents according to the morphology and conduction velocity of the axon and fall into three main categories (Erlanger & Gasser, 1937): C-fibres, are small diameter, unmyelinated fibres with conduction velocities of $<2 \text{ ms}^{-1}$; A δ -fibres, are myelinated fibres of a slightly larger diameter and conduction velocities of between 3 and 30 ms^{-1} ; and A β -fibres that are large diameter myelinated afferents with conduction velocities of 35-100 ms^{-1} . These studies also proposed that innocuous stimuli was

transmitted by the larger A β fibres and painful sensation from noxious stimuli was transmitted by thinly or non-myelinated A δ and C-fibres (Erlanger & Gasser, 1937; Heinbecker *et al.*, 1932; Zotterman, 1933). However, the hypothesis that A δ and C-fibres played a role in the transmission of nociceptive signals from peripheral tissue was not shown conclusively until the late 1960s by Perl and colleagues.

Using electrophysiological recordings from thinly myelinated A δ fibres innervating cat skin it was found that a small proportion had response thresholds to thermal and mechanical stimuli many times higher than that seen in other A δ fibres. These fibres, termed nociceptors, only responded to noxious thermal stimulation or intense mechanical stimulation through pinching of the skin (Burgess & Perl, 1967). Furthermore, in studies carried out on thin, unmyelinated C-fibres innervating the hind limb of the cat approximately half the afferents responded only to innocuous mechanical stimulation, with the rest responding only when the stimuli imposed were noxious and were therefore deemed nociceptors. The 50% of C-fibre nociceptors could be further divided with 80% responding to intense mechanical stimulation, noxious heat, and chemical irritants (these were named polymodal nociceptors), and 20% responding to high threshold mechanical stimulation alone (termed mechanonociceptors) (Heppelmann, 1997; Heppelmann *et al.*, 1988; Hildebrand *et al.*, 1991; Bessou & Perl, 1969). Polymodal nociceptors, because they make up the largest proportion of nociceptors and possess many unique properties including a high degree of plasticity, have been the subject of the most research.

There are two important peripheral mechanisms by which nociceptors can be activated, excitation and sensitisation. Excitation is the induction or increase of afferent neural discharge following stimulation (e.g. electrical, mechanical, thermal,

or chemical stimuli). Sensitisation or hyperalgesia produces a decrease in the threshold required to activate the nociceptor and an increase in the response to stimulation.

EXCITATION OF NOCICEPTORS

Activation of nociceptors results from depolarisation of peripheral terminals through activation of specific receptors. These receptors mediate the membrane permeability to a number of ionic species such as sodium (Na^+), calcium (Ca^{2+}), and potassium (K^+). An influx of Na^+ or Ca^{2+} into the cell in response to receptor activation cause a depolarisation of the cell membrane and triggers an action potential that is transferred to the CNS where it can be perceived as pain (Belmonte & Cervero, 1996).

Mechanical excitation

Afferents that respond to low intensity mechanical stimulation (mechanoreceptors) in the joint have conduction velocities in the $\text{A}\beta$ range and respond to normal movements. They have proprioceptive functions to determine the position of the joint in space as well as vibration within the structure (Schaible & Schmidt, 1988). Movements that are beyond the normal range, or applied force above a certain threshold are perceived as painful and these stimuli activate a separate population of $\text{A}\delta$ fibres known as mechanonociceptors. Very little is known about the specific mechanisms involved in the depolarisation of the nociceptor due to high-intensity mechanical stimulation. Work in non-mammalian systems such as the mechanosensitive channel of large conductance (MscL) in *Eschericia coli*, a channel that opens in response to a deformation of the lipid bi-layer (reviewed in Sukharev *et*

al., 1997), or the genetic manipulation of the nematode *Caenorhabditis elegans*, possessing a mechanosensitive sodium channel that can be blocked by amiloride (reviewed in Tavernarakis & Driscoll, 1997), point to the possibility of a specific mechanosensitive channel present on peripheral nociceptors, however to date this remains unknown.

Thermal excitation

There are two types of receptors in the skin that will respond to mild changes in temperature. Cold receptors have conduction velocities in the A δ to C-fibre range and discharge spontaneously at normal skin temperatures; discharge increases with cooling by as little as 0.5°C (Darian-Smith *et al.*, 1973). As with mechanoreceptors under normal conditions, these afferents appear to be insensitive to chemical stimulation, including capsaicin (Szolcsanyi *et al.*, 1988). Warm receptors conduct almost exclusively in the C-fibre range and are also spontaneously active at normal skin temperature (~30°C) and show linear increase in discharge as skin temperature increases, up to 46°C. Outside these constraints the afferents become silent (Darian-Smith & Johnson, 1977a; Darian-Smith & Johnson, 1977b).

Although mechanoreceptors can be excited by normally innocuous stimuli during injury or inflammation, cold and warm receptors do not appear to become sensitised. During inflammation, an increase in sensitivity to temperature is not the result of a recruitment of normally silent afferents but is instead mediated by alterations to nociceptive afferents in which their threshold to activation becomes lower (Meyer *et al.*, 1996).

Temperatures outside the normal physiological distribution are perceived as painful and these signals are transmitted by thermonociceptors. Very little is known about the mechanisms for the transduction of noxious cold stimuli, however in recent years the mechanism by which high temperatures stimulate nociceptors has begun to be explained. Capsaicin (8-methyl-N-vanillyl-6-noneamide) is present in chilli peppers and is the compound responsible for making them taste hot. With the discovery and cloning of the first vanilloid receptor (VR₁) a channel has been found that is gated by capsaicin and which is also sensitive to high temperature – that greater than 42 °C (Caterina *et al.*, 1997). A second channel, also found to be gated by noxious heat, the vanilloid receptor like channel (VRL-1) was cloned soon after. This channel, although from the same receptor family, is not sensitive to capsaicin and is not activated until temperatures exceed 53°C (Caterina *et al.*, 1999). For a detailed review of the properties of the vanilloid receptors see Szallasi & Blumberg (1999).

Chemical excitation

Peripheral nociceptors are capable of being activated by some chemical irritants acting through specific receptors to produce an acute painful response. However, during inflammation, a large number of chemical mediators are released that are able to directly activate, or in some cases, sensitise peripheral nociceptors producing a long lasting, chronic pain. The mechanisms for chemical activation and/or sensitisation of nociceptors will be examined in more detail below.

SENSITISATION OF NOCICEPTORS

In order to study changes that occur in nociceptor sensitivity it is easiest to

examine the changes in afferents innervating the skin, in response to damage done to tissue through injury or inflammation induced by noxious heat, local electrical stimulation or chemical irritants. There are two distinct zones that surround the site of injury in which the sensitivity of nociceptive afferents is altered. The first zone immediately surrounds the site of injury or inflammation. Sensitisation in this area is known as primary hyperalgesia. Hyperalgesia is defined as a state of increased pain sensation where a normally noxious stimulus is perceived as being more painful, and is characterised by spontaneous pain and an increased sensitivity to thermal or mechanical (and in the joint, chemical) stimuli (Hardy *et al.*, 1952). The second zone arises in the surrounding area and shows an increased sensitivity to mechanical but not thermal stimulation. The increased sensitivity means that normally non-noxious stimuli are now capable of producing pain – known as allodynia (Meyer *et al.*, 1996).

Increased sensitivity is not limited to the skin as similar responses are seen in other nociceptors, including those that innervate the rat knee joint (Heppelmann & Pawlak, 1997a; Heppelmann & Pawlak, 1997b; Kelly *et al.*, 1995a; Mapp *et al.*, 1996). The basis of this afferent sensitisation arises from the release of a number of pro-nociceptive mediators during inflammation that are capable of lowering the activation threshold of peripheral nociceptors.

Sensitisation is not restricted to the periphery. Neurons in the dorsal horn that receive projections from the DRG can also be sensitised producing a “wind-up” effect, which acts to increase the gain of the afferent inputs (Kress & Reeh, 1996). However, this thesis will focus on peripheral afferents and in particular the terminals of afferent nociceptors under inflammatory conditions.

ARTHRITIS

Arthritis can be divided according to primary pathology into two major categories, osteoarthritis and rheumatoid arthritis. Osteoarthritis (OA) is a slowly progressing degenerative disease that affects the major synovial joints primarily, but not exclusively, through non-immune processes. The condition comprises a number of vague clinical syndromes and is primarily defined by its associated symptoms, which include joint enlargement, deformity, stiffness, reduced function and a general decrease in the quality of life due to chronic pain. OA is typically associated with a particular joint and is usually the result of “wear and tear”. Joint instability as a result of a specific injury or a series of small insults can lead to a local inflammation in the joint. The inflammation is self-perpetuating and can lead to further joint destruction including the erosion of articular cartilage, and bone erosion and formation. The chronic inflammation and oedema in the synovial space can lead to severe pain through stimulation of peripheral nociceptors present in and around the joint (Schaible & Schmidt, 1996).

Rheumatoid arthritis (RA) is a systemic condition of unknown aetiology whose primary clinical feature is a chronic inflammation present in multiple joints (polyarthritis). In synovial joints the inflammation is symmetrical with joints on both the left and the right sides of the body being affected. One of the hallmarks of RA is the progressive destruction of the articular cartilage and erosion of bone due to the chronic inflammation present in the joint (Figure 1.1). RA is considered to be an autoimmune condition with the possibility of a number of different primary stimuli. One particular stimulus is the loss of tolerance to self-antigens existing on articular cartilage, therefore these auto-antigens become targets for the immune system

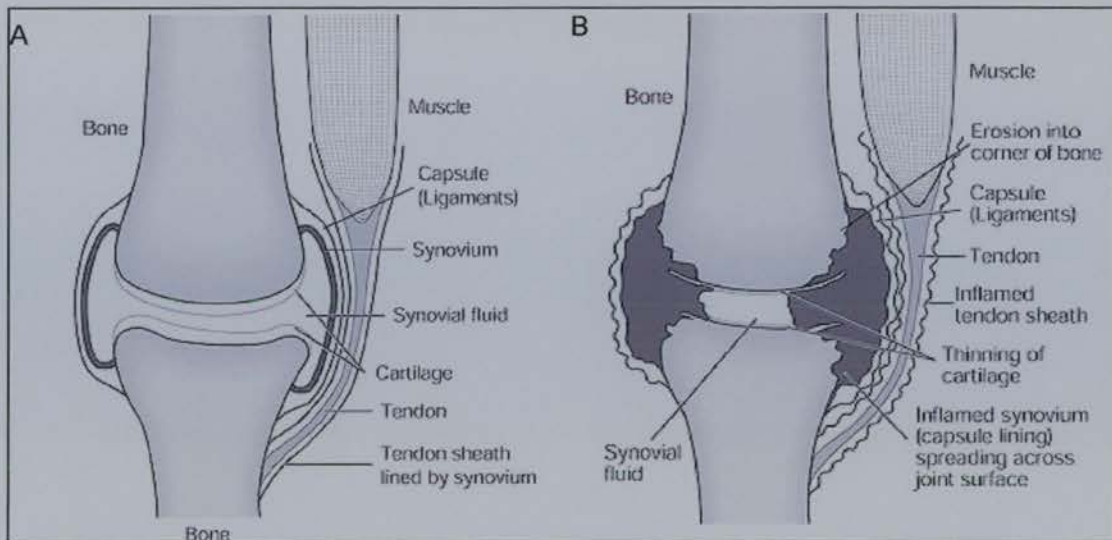


Figure 1.1 – Comparison of normal and arthritic knee joints

A schematic representation of A) normal and B) arthritic (rheumatoid) knee joint. The normal joint shows a thin synovial lining and smooth surfaces on the bone and overlying cartilage. The arthritic joint displays thickening of the synovial membrane (hypertrophy) due to invasion of inflammatory cells, which also infiltrate the joint space and surrounding tissues, including ligaments, tendons, and muscle (peri-articular inflammation). A combination of synoviocytes and polymorphonuclear leukocytes (PMN) overgrow the joint surface (pannus) and erode the cartilage and bone through the release of metalloproteinases and components of the complement system. Adapted from (Arthritis-Research-Foundation, 2002)

causing a localised and chronic inflammatory condition in the synovial space (Kelley *et al.*, 1997). In a normal joint the synovial lining consists of a very thin membrane only a couple of cells thick, isolating the largely acellular joint space. During arthritis the synovial inflammation, or synovitis, involves a very significant thickening of the synovial membrane as well as the invasion of inflammatory cells (consisting mostly of polymorphonuclear (PMN) leukocytes) into the synovial membrane and the joint space. One of the characteristic features of RA is the formation of pannus. Pannus is the tissue formed at the boundary of the cartilage and the synovial lining and consists

of both synoviocytes and inflammatory cells. The pannus overgrows the healthy cartilage starting from the border with the synovium. Within the pannus tissue a number of mediators are released including neuropeptides and cytokines that perpetuate the inflammation, and oxygen radicals, and lysosomal and metalloproteinase enzymes, that cause cartilage destruction and bone erosion (Kelley *et al.*, 1997). Standard treatments for chronic inflammatory conditions, such as RA, still focus on reducing inflammation, in an attempt to treat the associated pain.

In order to examine RA in an experimental setting a number of animal models of the condition have been developed. Although no one model provides perfect mimicry of the clinical condition they can provide valuable insight into the disease process. In this thesis two models of induced experimental arthritis in the knee joint, one in the rat (Chapter 3) and one in mouse (Chapter 6), will be discussed and used. The knee joint provides a well-characterised environment in which to study the responses of peripheral nociceptors under normal conditions, and during chronic joint inflammation.

KNEE JOINT ARTICULAR AFFERENTS

There is an extensive literature on sensory nerve innervation in a wide variety of tissues throughout the body including skin (Bessou & Perl, 1969; Burgess & Perl, 1967; Torebjörk & Hallin, 1974), joints (Heppelmann, 1997; Mapp, 1995; Schaible & Schmidt, 1983b; Schepelmann *et al.*, 1992), cornea (Belmonte *et al.*, 1991; Belmonte & Giraldez, 1981; Giraldez *et al.*, 1979), skeletal muscle (Mense & Meyer, 1985), airways (Fox *et al.*, 1993), viscera (Cervero, 1994), and teeth (Greenwood *et al.*, 1972). Only knee joint sensory nerves will be described due to their relevance to the experimental work undertaken. Furthermore, although the knee joint is

innervated by both myelinated and unmyelinated afferents as well as unmyelinated sympathetic efferents (Freeman & Wyke, 1967; Polacek, 1966; Skoglund, 1956) it is the articular afferents and in particular articular nociceptors that are the focus of this thesis.

Anatomy of articular afferents

Early anatomical looking at the innervation of the knee joint were done in the knee joint of the cat (Freeman & Wyke, 1967; Gardner, 1944; Skoglund, 1956). To date the cat knee joint has been the most extensively examined, however, studies in other species including rats (Hildebrand *et al.*, 1991) and humans (Biedert *et al.*, 1992; Halata *et al.*, 1985; Wojtys *et al.*, 1990) have shown that the pattern of innervation, types of fibres, and ratio of myelinated to unmyelinated fibres are broadly similar between species with differences found only in the absolute number of fibres innervating the joint, which is dependent on the size of the animal.

The articular branches that exit the saphenous nerve on the medial aspect of the limb supply the knee joint – see Figure 1.2. The innervation arises primarily from two articular nerves, the posterior articular nerve (PAN) and the medial articular nerve (MAN). In this thesis, electrical recordings of sensory nerve activity were recorded from the MAN, therefore further discussions will be restricted to this articular nerve.

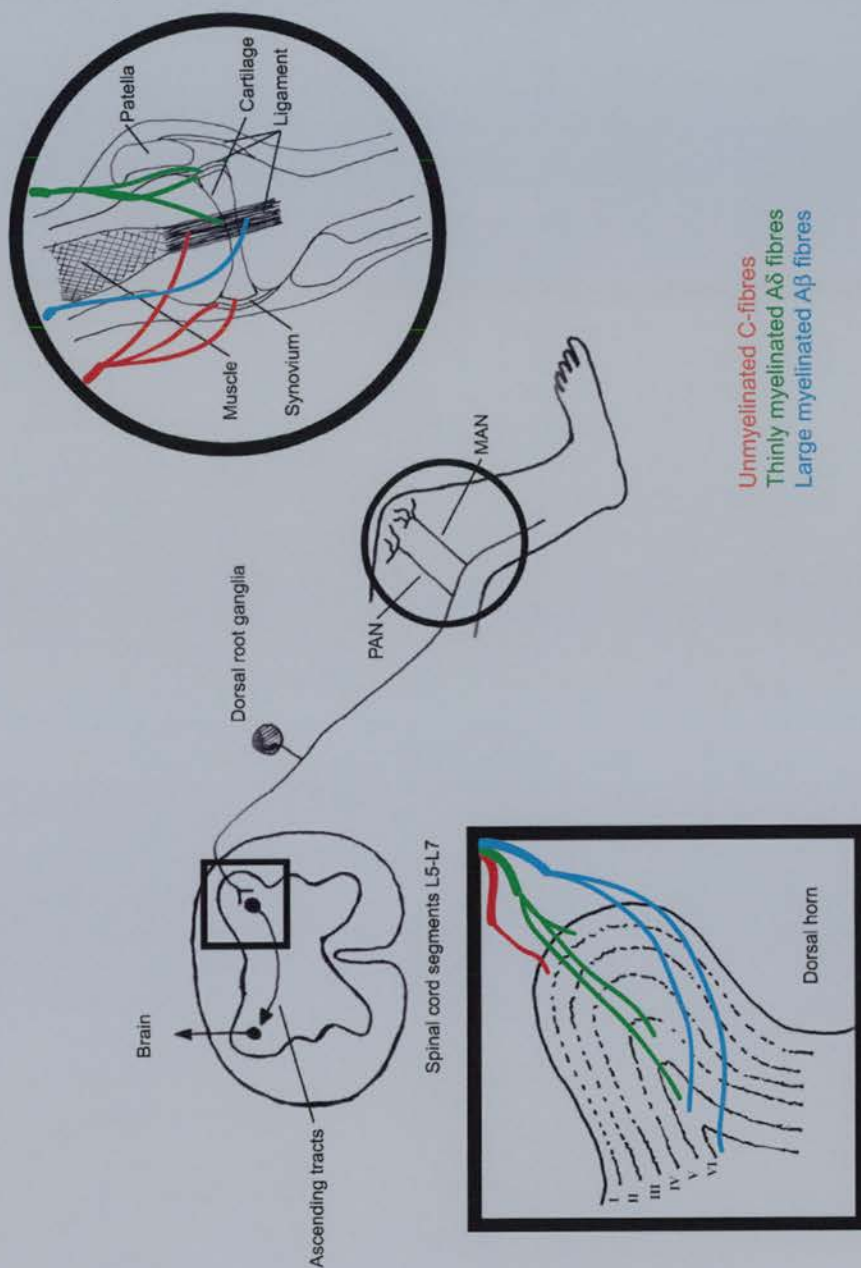


Figure 1.2 – Afferent innervation of the knee joint

Schematic representation of afferent innervation of the knee joint. Terminals of fine, unmyelinated C-fibres, thinly myelinated Aδ fibres, and large myelinated Aβ fibres ending finely branched “free endings” within the joint capsule, synovium and ligaments. Axons for these fibres branch from the main saphenous nerve via the medial and posterior articular nerves (MAN and PAN respectively). The cell bodies for these fibres are in the L5 and L6 dorsal root ganglia (DRG) and from there they also project centrally into the dorsal horn (DH) of the spinal cord where they have their first synapse.

The MAN branches from the saphenous nerve and traverses the antero-medial aspect of the thigh and runs alongside the descending genicular artery and vein. At the medial aspect of the knee joint the nerve divides into two branches and spreads out to innervate the medial and antero-medial areas of the fibrous joint capsule including the articular ligaments, menisci and adjacent periosteum, and adipose tissue (Freeman & Wyke, 1967; Heppelmann *et al.*, 1990; Johansson *et al.*, 1991; Polacek, 1966). These studies, using both light and electron microscopy, were unable to find sensory innervation in the synovium. However, recent studies have shown that the release of inflammatory mediators including substance-P, somatostatin, and calcitonin gene related peptide (CGRP) in the synovium is from afferent terminals leading to the supposition that terminals are present (Ferrell & Russell, 1985; Imai *et al.*, 1997).

Much of the literature on knee joint afferents utilises a classification system dividing the fibres into four types (Group I-IV) based on their morphology (for review see Schaible & Grubb (1993). However, to avoid confusion the Erlanger and Gasser classification system (A β fibres, A δ fibres, and C fibres) will be used for the remainder of this thesis.

The MAN of the cat contains approximately 630 afferent fibres and the majority of these fibres are A δ fibres and C-fibres with only approximately 20% of the fibres being classified as A β fibres (Heppelmann, 1997; Heppelmann *et al.*, 1988; Hildebrand *et al.*, 1991; Langford & Schmidt, 1983). Although a small proportion of afferent fibres innervating the knee joint respond to low threshold mechanical stimulation (A δ 35%, C 10%) the vast majority of C-fibres and approximately 30% of A δ fibres are only excited by noxious movements of the joint or are insensitive to

mechanical stimulation under normal conditions (Schaible & Grubb, 1993).

From their peripheral terminals, articular afferents extend long axons up to the cell bodies in the dorsal root ganglia (DRG). The size of the cell body of neurons within the DRG is correlated with the type of afferent fibre, with C-fibres having small diameter cell bodies (0.4-2.0 μm), A δ fibres having a medium diameter somas (2-6 μm), and A β fibres having the largest diameter cell bodies (>10 μm) (Millan, 1999). Since the majority of cutaneous and articular afferents are C-fibre polymodal nociceptors, it is assumed that the properties of small diameter neurons relates to nociceptors.

In addition to sending axons to the periphery, articular nociceptors project axons from the DRG into the dorsal horn of the spinal cord. Using retrograde transport of horseradish peroxidase (HRP) as a tracer it has been shown in the cat knee joint the afferents of the MAN enter the spinal cord via L5 and L6 (Craig *et al.*, 1988; Skoglund, 1956). Once in the dorsal horn these afferents have projection fields that are limited to the cap of laminae I, laminae V-VI, and the dorsal part of laminae VII (Craig *et al.*, 1988).

The anatomy of articular nociceptors is such that compounds that modulate their function can act a number of different sites both centrally, in the dorsal horn, and peripherally, in the DRG and at peripheral terminals. The focus of this thesis is the peripheral terminals of articular nociceptors. The terminal is the point at which a nociceptive signal is initiated and as such interference at this point would eliminate the central processing of the input and the perception of pain. Furthermore, the wide array of pharmacological targets at the peripheral terminal allows for many different therapeutic approaches to be used (Figure 1.3). Finally, by focusing on the periphery

INFLAMMATORY MEDIATORS IN NOCICEPTION

A normal joint is painful only when exposed to extreme external stimuli such as over-rotation or a strong external pressure. In the diseased state nociceptive afferents innervating the joint become sensitised and the joint can be painful during normal movement, or even while resting. The normal responses of nociceptors are altered during chronic inflammation by the release of a large number of mediators that originate from neural and non-neural sources. Neural sources involve the release of substances from the peripheral terminals of both postganglionic sympathetic efferents and sensory afferents. Additionally, as with all inflammations, a large number of immune cells are recruited to the site such as macrophages, polymorphonuclear leukocytes, and mast cells, all of which release mediators into the surrounding tissue.

Regardless of whether substances released during inflammation originate from the nervous or immune systems there are two mechanisms through which mediators can affect peripheral nociceptor function, *directly* on the sensory nerve terminal to excite it, or *indirectly* by triggering the release of another algogenic mediators and/or sensitising the afferent to activation by another stimulus.

Directly acting algogenic mediators

Algogenic chemicals that directly activate nociceptors do so by activating specific receptors on the afferent terminals. Some compounds such as capsaicin, and adenosine 5' triphosphate (ATP) produce a rapid depolarisation of the nerve by activating specific ligand-gated ion channel linked receptors causing a rapid influx of ionic species. Other compounds bind to receptors that are linked to guanyl-

nucleotide-binding proteins (G-proteins) that cause depolarisation via intra-cellular second messenger systems. The algogens bradykinin, ATP, and capsaicin are of particular relevance to this thesis and as such will be discussed in detail below.

Bradykinin (BK)

Tissue damage, inflammation, and an acidic environment will trigger the activation of a group of proteolytic enzymes, one present in the plasma, the other widely distributed in a number of different tissues, known as kallikreins, that are responsible for the production of the nonapeptide bradykinin (BK: **Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg**) from its precursor (Marceau, 1995). Bradykinin, in addition to its role in mediating vascular permeability, has long been shown to be a potent activator of peripheral nociceptors (Keele & Armstrong, 1964). The effects of bradykinin are mediated through two G-protein coupled receptors, namely bradykinin receptor B1 and B2. The use of specific receptor antagonists has established that the B2 receptor is constitutively expressed in both the DRG (Steranka *et al.*, 1988) and peripheral terminals (Kelly *et al.*, 1995a). The bradykinin B2 receptor plays a more critical role in acute inflammation – including inducing oedema and pain, whereas the inducible B1 receptor is up-regulated during conditions of persistent inflammation and is likely to play a role in chronic pain (Hall, 1997).

Bradykinin is released in response to the pro-inflammatory cytokines IL-1 β and TNF- α (Dray & Perkins, 1993; Dray & Urban, 1996) and it acts both as a pro-inflammatory mediator and an algogen. Injection of bradykinin induces inflammation in both animals and humans by acting at the B2 receptor (Marceau *et*

al., 1983), and circulating levels of bradykinin have been shown to be 3-fold higher in patients with RA (Hargreaves *et al.*, 1988). In addition, bradykinin is able to activate C and A δ nociceptors and can induce pain in humans when applied to blister bases where it was shown to be one of the most potent algesics known (Whalley *et al.*, 1987). Bradykinin activates peripheral nociceptors innervating the knee (Kanaka *et al.*, 1985; Kelly, 1998; Kelly *et al.*, 1995a) and the ankle joint in the rat (Asghar *et al.*, 1997). Furthermore, a specific B2 receptor antagonist blocks the hyperalgesia associated with inflammation (Perkins *et al.*, 1993; Perkins *et al.*, 1995) and transgenic mice lacking the B2 receptor showed no response to an intra-plantar injection of carrageenan (Rupniak *et al.*, 1997) demonstrating a crucial role for bradykinin in peripheral nociceptor sensitisation.

Adenosine 5'-triphosphate

Adenosine 5'-triphosphate (ATP; Figure 1.4) is a ubiquitous cytoplasmic constituent and is present at high levels when released from damaged cells at an injured or inflamed site, as is confirmed by the increased concentration of ATP in the synovial fluid of patients with rheumatoid arthritis (Park *et al.*, 1996; Ryan *et al.*, 1996). Since ATP was able to evoke pain when applied to blister bases it was proposed that the nucleotide could act as a neuronal activator and play an important role in pain signalling (Bleehen *et al.*, 1976; Bleehen & Keele, 1977). For extracellular ATP to behave as an activator of peripheral nociceptors it needs to act directly and have a mechanism for breakdown or reuptake. As there exists a well-described mechanism for the breakdown of ATP to adenosine via nucleotidases present in almost every tissue (Figueroa *et al.*, 1989; Nustad *et al.*, 1975) all that remained was to determine if activation of sensory nociceptors was non-specific, or

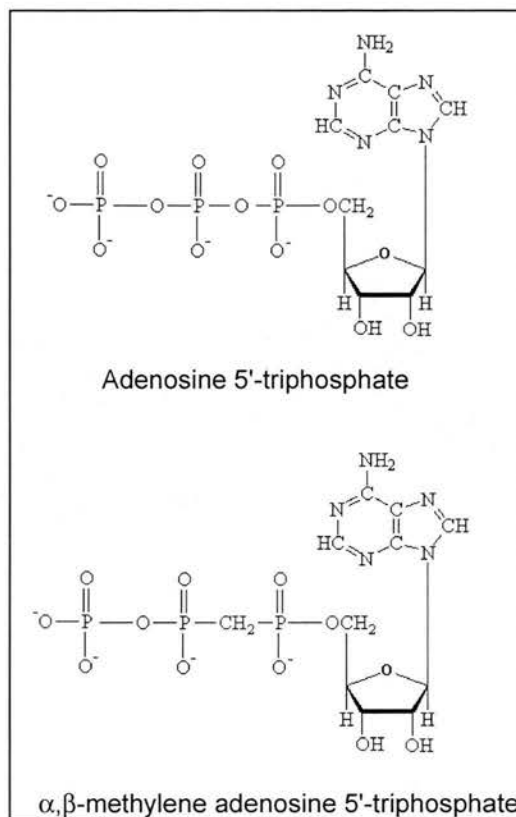


Figure 1.4 – ATP and its stable analog α,β -methylene ATP

Structure of ATP and α,β -methylene ATP ($\alpha\beta$ meATP). $\alpha\beta$ meATP is a stable analog that specifically activates P2X1 and P2X3 receptor subtypes.

receptor mediated.

The existence of an ATP receptor was first suggested by (Burnstock *et al.*, 1970) after proposing that it (and its metabolite, adenosine) was the substance released from autonomic nerves innervating intestinal smooth muscle, something previously shown in sensory nerves (Holton, 1959). It was proposed that these purine receptors be classified as P1-purinergic receptors for adenosine and P2-purinergic receptors for ATP (Burnstock *et al.*, 1978). Further research showed that ATP activates two types of receptors; ATP-gated ion channels (P2X receptors) and G-

Subtype	$\alpha\beta$ meATP sensitivity	Antagonist sensitivity	Desensitisation	Agonist potency ratio
P2X ₁	Yes	Yes	Rapid	2meSATP \geq ATP $>$ $\alpha\beta$ meATP $>>$ ADP
P2X ₂	No	Yes	Slow	ATP = ATP γ S = 2meSATP
P2X ₃	Yes	Yes	Rapid	2meSATP $>>$ ATP $>$ $\alpha\beta$ meATP
P2X ₄	No	No	Slow	ATP $>$ ATP γ S $>$ 2meSATP $>$ ADP = $\alpha\beta$ meATP.
P2X ₅	No	Yes	Slow	ATP $>$ 2meSATP $>$ ADP
P2X ₆	No	No	Slow	ATP $>$ 2meSATP $>$ ADP
P2X ₇	No	Partial	Slow	BzATP $>$ ATP $>>$ 2meSATP $>$ ATP γ S $>$ ADP

Table 1.1 – Pharmacological profile of P2X receptors

Functional characteristics of cloned P2X receptor subtypes expressed in *Xenopus* oocytes or mammalian cells. Modified from (Collo *et al.*, 1997).

Abbreviations: $\alpha\beta$ meATP, α,β -methylene ATP; 2meSATP, 2-methylthio ATP; ATP γ S, adenosine 5'-O-(3-thiotriphosphate; BzATP, 3'-O-(4-benzoyl)benzoyl ATP.

1997). In addition to the role of ATP in nociception it has also been proposed that activation of the P2X₇ receptor on mast cells and macrophages may be responsible for releasing of the pro-inflammatory cytokine IL-1 β which subsequently produces sensitisation of peripheral nociceptors – this is addressed in detail in Chapter 6.3.

Capsaicin

Capsaicin (8-methyl-vanillyl-6-nonenamide; Figure 1.5) is the active, pungent ingredient in chilli peppers. The topical application of capsaicin to the skin or to mucous membranes produces a painful, burning sensation (Keele & Armstrong,

1964). With prolonged exposure there is desensitisation of the response, and analgesia results (Szallasi & Blumberg, 1999). Capsaicin selectively activates a nociceptive population of C-fibres and causes depolarisation of small diameter sensory neurons in the DRG (Wood *et al.*, 1988). As capsaicin can specifically activate some nociceptors, the mechanism of action is likely to be an important target for the development of novel analgesics.

Caterina *et al.* (1997) took the first step towards the elucidation of the action of capsaicin by cloning the receptor on afferent nociceptors that responds to capsaicin. As capsaicin and its active analogs contain the vanillyl chemical moiety the receptor was designated the vanilloid receptor (VR₁). In addition to being activated by capsaicin, the VR₁ receptor is an ion channel that is gated by noxious heat, i.e. is activated by temperatures above 42°C (Caterina *et al.*, 1997). As capsaicin specifically activates noxious heat sensitive C-fibres, it is likely that the

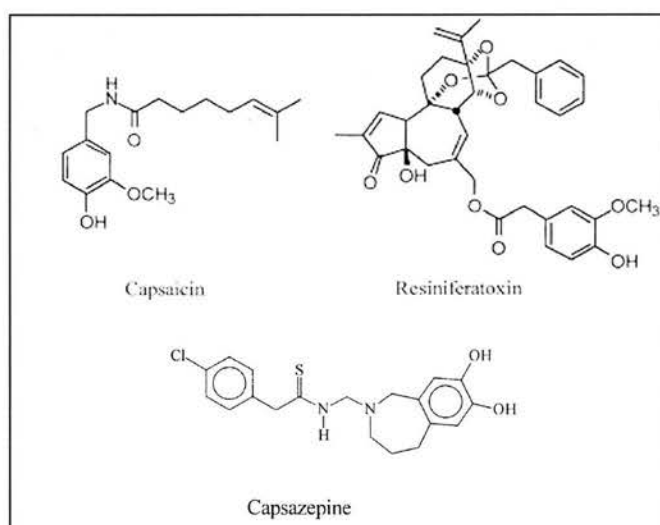


Figure 1.5 – Structure of VR₁ agonists and antagonists

Structure of VR₁ agonists capsaicin and resiniferatoxin. Capsazepine is a specific VR₁ antagonist.

VR₁ receptor plays an important role in nociceptive processing. For that reason a lot of effort has been put into finding an endogenous compound that chemically activates peripheral nociceptors via the VR₁ receptor. One potential endogenous ligand, anandamide, and its interaction with the VR₁ receptor will be addressed in this thesis (see Chapter 5).

Indirectly acting algogenic mediators

The presence of inflammatory mediators during chronic inflammation can drastically affect the responses of nociceptors innervating inflamed tissue. Nociceptors are silent under normal conditions however during inflammation afferents have an increased spontaneous discharge rate, a decreased threshold for activation, and a greater response to noxious stimuli than that seen in normal tissue. Furthermore, under inflammatory conditions afferents can show changes in the type of stimulus required to activate them, becoming susceptible to a much broader spectrum of stimuli (Coggeshall *et al.*, 1983; Guilbaud *et al.*, 1985a; Schaible & Schmidt, 1985) and in some cases afferents that are normally non-responsive, even to acute noxious input (silent nociceptors), start generating noxious signals (Schaible & Schmidt, 1996). For example, although there is no evidence that mechanoreceptors in the skin can be activated by thermal or chemical stimulation under normal conditions, during inflammation both skin and joint afferents become responsive and spontaneously active (Belmonte & Cervero, 1996).

Mediators released from immune cells

As stated above, the peripheral terminals of articular nociceptors are embedded into a wide variety of tissues and are open to stimulation by the immediate

environment. Therefore the terminal is the point at which the peripheral nervous system and the immune system readily come in contact. Immune cells drawn to the site of inflammation release a wide variety of pro- and anti-inflammatory cytokines, prostaglandins, thromboxanes and other molecules, all of which affect the surrounding tissue, and some of which are capable of altering the sensitivity of peripheral nociceptors. Joint afferents, under normal conditions can be sensitised and become spontaneously active or show an increased response to noxious stimuli after the local injection of prostaglandin I₂ (prostacyclin) (Birrell *et al.*, 1993; Schepelmann *et al.*, 1992), prostaglandin E₂ (Birrell *et al.*, 1990b; Grubb *et al.*, 1991), and interleukin 1 β (Kelly *et al.*, 1997).

Mediators released from sensory terminals

In addition to transferring information from the periphery, sensory afferents are also able to mediate their own function or the local environment by the release of active neuropeptides from peripheral terminals and into the circulation. The compounds produced in the neurons are neuropeptides and they are synthesised both in the cell body and transported to the terminal or at the terminal itself (for example (Heppelmann *et al.*, 1997) and are released upon stimulation. Using immunohistochemical techniques it has been shown that peptides are expressed in the small diameter neurons (primarily C-fibres) of the DRG under normal conditions and include substance-P, calcitonin-gene-related peptide (CGRP), preprotachykinin (PPT) and somatostatin (Donaldson *et al.*, 1995; Hanesch *et al.*, 1991; Ju *et al.*, 1987; Smith *et al.*, 1993).

Only a proportion of C-fibre afferents are polymodal nociceptors containing neuropeptides, which leads to a further division into peptidergic and non-peptidergic

afferents. In the rat DRG approximately 50% of small diameter neurons exhibit some sort of peptide immuno-reactivity (Hunt & Rossi, 1985). However as the DRG contains the cell bodies of all C-fibres innervating a wide variety of tissues it is difficult to determine if there is differential innervation of peptidergic fibres in specific tissues. Therefore, although the majority of fibres innervating the knee joint are polymodal C-fibre nociceptors, it is not currently known whether all C-fibres are peptidergic or whether there is a proportion that are non-peptidergic (Lawson, 1996). The classification of whether or not a neuron is peptidergic is based on the type of trophic factor that maintains its survival. Neurons that require nerve growth factor (NGF) express neuropeptides whereas those that require brain derived neurotrophic factor (BDNF) or glial cell line-derived neurotrophic factor (GDNF) do not express neuropeptides, but will bind the plant lectin, isolectin B4 (IB4) (Snider & McMahon, 1998). Recent findings by Guo et al (1999) examining the expression of the receptor for the potent C-fibre activator capsaicin (see below) in the rat DRG found that the receptor was only colocalised with a proportion of CGRP and SP positive cells. Instead the capsaicin receptor colocalised with the binding of IB4, placing it on non-peptidergic neurons (Guo *et al.*, 1999).

For the purpose of this study, two indirect mediators of nociceptor function studied. Both compounds are released from sensory terminals during inflammation however details of their specific actions are not detailed here but instead are reviewed in subsequent chapters dedicated to the individual mediators: the neuropeptide, somatostatin (see Chapter 4), and the endogenous cannabinoid, anandamide (see Chapter 5). In addition to investigating the effect of these compounds on nociceptive afferents, their ability to modulate the effects of potent,

known activators of peripheral nociceptors, including the algogens bradykinin, ATP, and capsaicin was also examined.

AIMS OF THE STUDIES

In this thesis predominantly electrophysiological techniques were used to examine the role of a number of mediators in the induction of chronic inflammation and activation of peripheral nociceptors in normal and arthritic knee joints in the rat and the mouse. The hypotheses that were tested are as follows:

- FCA-induced chronic inflammation increases the sensitivity of nociceptive afferents to responses evoked by the algogens capsaicin, bradykinin, and ATP.
- The neuropeptide somatostatin inhibits spontaneous and evoked excitation in afferents innervating the normal and arthritic rat knee joint.
- Anandamide, an endogenous cannabinoid-like substance, can activate peripheral nociceptors through its reported action at the vanilloid receptor (VR₁).

In recent years there have been enormous developments in the manipulation of the mouse genome. The manipulation of specific genes in the mouse means that targeting of a specific receptor is greatly facilitated and does not require the development of specific agonists and antagonists. To study transgenic mice with mutations of receptor types relevant to nociception and chronic inflammation, it was necessary to determine the feasibility of inducing a chronic inflammation in the mouse knee joint. Furthermore, to determine the role of the target gene in peripheral

nociceptive transmission the feasibility of electrophysiological recording of knee joint afferents was also examined. The aims of this thesis are:

- Develop a murine model of chronic unilateral joint inflammation that is consistent across a number of different mouse strains.
- Determine the role of the purinoceptor P2X₇ in the establishment and maintenance of chronic joint inflammation.
- Develop a method for the electrophysiological recording of evoked responses from peripheral afferents innervating the mouse knee joint.

2. Methods

The experiments detailed in this thesis were performed in accord with the regulations and guidelines of the Scientific Procedures Act (1986) under the Personal Licence number (PIL): 60/7128 and Project Licence number (PPL): 60/2750. Animals used in this study were housed in a licensed animal facility within the Faculty of Medicine and experiments were performed in licensed premises within the Department of Neuroscience (formerly Department of Pharmacology) at the University of Edinburgh.

In this present study a total of 154 male Wistar rats, 104 male DBA-1 mice, 12 female DBA-1 mice, and 76 male C57BL6 mice were used. All animals were purchased from Charles River (UK), with the exception of the DBA-1 mice which were purchased from Harlan (UK). Also included in this study were 12 transgenic male DBA-1 mice and 12 wild type littermates received from GlaxoSmithKline (UK). Histological studies on tissue taken and fixed at the University of Edinburgh were performed at GlaxoSmithKline (Ware, UK). Animals were kept on a 12-hour light/dark cycle and maintained on standard animals feed and water *ad libitum* for the duration of all experiments. All in vivo experiments were performed between 9.00 and 17.00.

2.1 MODELS OF EXPERIMENTAL ARTHRITIS

RAT MODEL OF UNILATERAL ARTHRITIS

Induction of unilateral arthritis

A localised chronic inflammation was induced in rats (180-200 g) by intra-articular (i. art.) injection of Freund's Complete Adjuvant (FCA, 1 mg ml⁻¹ heat

killed *Mycobacterium tuberculosis* suspended in heavy liquid paraffin oil) into the left knee (stifle) joint under transient halothane anaesthesia (3% in oxygen). The knee was swabbed with 70% ethanol and the FCA (150 µl) was injected using a sterile 26-gauge needle (Microlance, UK) inserted into the joint space through the patella tendon just below the patella. Once the animals had recovered from anaesthesia they were housed up to four in a cage until they were used. Control animals were injected with heavy liquid paraffin oil (HLP).

Assessment of arthritis

The single intra-articular injection of FCA produces a mild to moderate chronic inflammation that reaches its peak after approximately 14 days and persists for up to 100 days, which is the time limit on our Project Licence. During this time the general health of the animals remains good as shown by their ability to gain weight, groom and move around as normal (Donaldson *et al.*, 1993). Animals were used for electrophysiological studies 14-35 days following the induction of arthritis. The general condition of the animals was monitored for discomfort and mobility throughout this time by observation (either by myself or the Named Animal Care Worker). The level of inflammation and allodynia associated with the experimental arthritis was determined prior to their being used in neural experiments. Within the laboratory the induction of unilateral arthritis has been shown to produce a mild inflammation, however procedures were in place for animals experiencing severe discomfort to be quickly and humanely killed either by myself or the Named Animal Care Worker.

Inflammation

The extent of joint inflammation was established by measuring the width of the joint at the middle of the joint capsule (diameter) of both the injected and uninjected joint using microcalipers (Mitutoyo, Japan). The induced inflammation was restricted to the injected joint, showing no spread to the contralateral joint, therefore the uninjected joint acted as an internal control and inflammation could be expressed as the increase in joint diameter of the arthritic (left, injected) versus the normal (right, uninjected) knee.

Allodynia

The inflammation-associated allodynia was determined by comparing the distribution of body weight between the normal and arthritic limbs using a dual channel weight averager (GlaxoSmithKline/Linton Instruments; (Clayton *et al.*, 1997). Briefly, the instrument consists of a pair of force transducers that were capable of measuring the weight applied on each limb over a fixed time (7 seconds). The rats were restrained for a short period of time, in a clear Perspex box, which required them to stand with each hind limb on a plate connected to the transducer. The force on each transducer was measured over the fixed time and given as a digital readout in grams applied by each limb. Given the internal control, the result could be expressed as the decrease in weight applied to the arthritic versus the normal limb i.e. the rat will distribute the body weight such that the inflamed limb bears less weight than the normal limb.

MURINE MODEL OF UNILATERAL ARTHRITIS

As the development of this novel model comprised a significant proportion of the overall project, it makes up a separate chapter in this thesis. Full details on the methods are provided in chapter 6.

2.2 IN VIVO RECORDING FROM AFFERENT NERVES

RECORDING FROM AFFERENT NERVES IN THE RAT KNEE JOINT

Anaesthesia and surgical procedures

Rats were initially anaesthetised with an intra-peritoneal (i.p.) injection of pentobarbitone (60 mg kg^{-1}). Once fully anaesthetised the rat was laid on its back and its core body temperature maintained at 38°C using an automated heating blanket (Harvard Apparatus Limited, UK) attached to a thermistor probe inserted into the rectum. A small midline incision was made in the neck to expose the trachea which was cannulated (cannula outer diameter (OD): 2.0 mm, Portex, UK) to allow for the spontaneous breathing of room air or, if required, artificial respiration using a ventilator (Harvard Apparatus Limited, UK). The right carotid artery was cannulated (OD: 0.75 mm) to allow for the continual monitoring and recording of arterial blood pressure through a pressure transducer (Bell and Howell, UK) attached to computer (MacLab/8 linked with a Macintosh LC475). Deep anaesthesia and fluid levels were maintained throughout the experiment with an intravenous (i.v.) infusion of pentobarbitone ($0.4\text{--}0.5 \text{ mg kg}^{-1} \text{ min}^{-1}$) diluted 50:50 in saline administered through a cannula (OD: 0.63 mm) inserted into the right femoral vein exposed after opening the medial aspect of the right hind limb. An additional cannula (OD: 0.70 mm) was

inserted into the right femoral artery with its tip positioned in the lower abdominal aorta to allow for the close intra-arterial (i.a.) of drugs to the left knee joint. Experiments could last upwards of 6 hours therefore changes in blood chemistry (pH, pCO₂, and pO₂) were periodically monitored.

Dissection of the medial articular nerve

Extracellular recordings were performed on a portion (filament) of the medial articular nerve (MAN) innervating the left knee joint. The left hind limb was fixed to a support using plaster of Paris and a small incision was made on the medial aspect in order to expose the MAN where it leaves the saphenous nerve. The skin was secured to a small brass ring to form a pouch that was filled with heavy liquid paraffin oil (HLP) to create an electrically isolated system. The saphenous nerve was then cut centrally to prevent interference from efferent neural activity. Furthermore, input from skin afferents was restricted by separating as much of the skin as possible from the knee joint, thereby rupturing any afferents innervating the skin. The MAN was dissected from the surrounding tissue and was split using fine forceps until a small filament, typically containing 1 – 4 functional afferent fibres was isolated. Once isolated, electrical activity was recorded using a bipolar platinum/iridium (Pt/Ir) that is placed half way between the saphenous nerve and the joint capsule (see Figure 2.1B).

Recording from the medial articular nerve

Afferent activity was recorded by laying the dissected MAN fibres over bipolar Pt/Ir wire electrodes (Figure 2.1). The raw nerve signal was passed through both a pre-amplifier and an amplifier (Neurolog NL 103 and NL 105 respectively;

signal amplified x 10 000). It was then displayed on an oscilloscope (Gould 1604), digitised (Sony, Digital Audio Processor PCM-701ES), and stored on videotape (Sony, Hi8 Recorder EV-C2000E PAL). The signal was then filtered - 100Hz low pass and 1000 Hz high pass (Neurolog, NL 115), and passed through a voltage discriminator (Digitimer D.130) connected to a loud speaker, before being displayed on the oscilloscope. For the purpose of off-line data analysis, both the filtered and the voltage discriminated signal passed through a Micro1401 interface (Cambridge Electronic Design, UK) connected to a personal computer (Research Machines, Pentium III/500 MHz) running Spike2 software (Cambridge Electronic Design, version 3.20). Electrical events were recorded on-line by setting both positive and negative trigger levels. These were saved as data files for off-line analysis. In addition keyboard markers were written into the file while recording to show the exact point at which the drugs were injected. Figure 2.2 illustrates the experimental set-up.

RECORDING FROM AFFERENT NERVES IN THE MOUSE KNEE JOINT

As with the development of the unilateral arthritis model mouse, the techniques developed for the recording from the mouse knee joint represent a significant component of the work undertaken for this thesis, and full details on the final procedures involved are provided in Chapter 6.2

IDENTIFICATION OF FIBRES

To determine the types of afferent fibres recorded within the whole filament, individual fibres were characterised according to their mechanosensitivity, conduction velocity, spike duration and chemosensitivity.

Mechanosensitivity

Probing the knee joint using a hand held plastic probe identified the receptive field being recorded as well as the receptive field of the individual fibres and their mechano-sensitivity. The tip of the probe was approximately 1 mm in diameter. The receptive field was only identified for the medial aspect because of the restricted access to the whole knee joint, and consequently the mechanosensitivity of some afferents showing spontaneous activity could not be confirmed.

Conduction velocity

Conduction velocities were not done routinely to prevent the sensitisation of the preparation following repeated electrical stimulation. However, where possible conduction velocities of afferents were determined using a stimulating electrode made up of a silver wire core insulated within a metal tube (external diameter: 1mm). This electrode was placed onto receptive field of a particular afferent as identified using the plastic probe. Supra-threshold 1 ms (0.3 - 5 V) pulses were delivered at a frequency of 1 Hz in order to trigger firing in the afferent, and by measuring (ruler) the distance between the recording electrode and the stimulating electrode, an estimate of the conduction velocity (CV) was made:

$$CV(m/s) = \frac{d(mm)}{t(ms)}$$

Where d is the distance in mm and t is the time in ms.

Spike duration

Data was collected and analysed off-line using Spike2 software. Within the software is the capability of measuring the duration of action potentials produced by individual fibres. Action potentials of short spike duration (<1.5 ms) were taken to be A δ mechanonociceptors and those with a longer duration (>2 ms) were characterised as C-fibre polymodal nociceptors (Gee *et al.*, 1999; Iggo, 1978).

Chemosensitivity

The afferents innervating the knee joint show a heterogeneous response to a number of algogens (Dowd, 1999). Therefore where possible the chemosensitivity to the algogens capsaicin (9 nmol), bradykinin (9 nmol), and $\alpha\beta$ meATP (60 nmol) were administered (see below) to establish a pharmacological profile of these afferent terminals.

DRUG ADMINISTRATION

Drugs were administered by a close intra-arterial (i.a.) bolus injection via the femoral, arterial cannula over 2s. Drugs were injected in a total volume of 0.1 ml and washed in with 0.2 ml saline (0.9% w/v NaCl in distilled water) as the catheter dead space was 0.1 ml. As the cannula tip had been positioned in the abdominal aorta, the injected compound was rapidly (within 2s) carried by the circulation into the (uncannulated) contralateral hind limb from which neural recordings were made. To prevent desensitisation of the preparation or a particular receptor 20 minutes was left between drug injections.

With this method it is not possible to get an exact measurement of the local drug concentration at the peripheral terminals. Therefore, the amount of drug is

stated as moles injected and, although it is appreciated that the entire amount is unlikely to reach the site of action. The drugs used in this study, including molecular mass, and suppliers are given in Appendix I.

DATA SORTING

Once the experiment was recorded on the computer it was analysed using Spike2 software. The recorded waveform was scanned and the program templates used to represent the individual spikes that make up the recorded response. Once this had been done the waveform was rescanned and the response of each fibre displayed on separate channels. These were generally displayed in histogram format, showing the total number of impulses in 1 s bins to provide both the absolute discharge (impulses) and the frequency (impulses s^{-1}).

DATA ANALYSIS

Drug effects were determined by comparing the action potential discharge frequency or the absolute number of action potentials recorded immediately following injection (test period) for the whole duration of the response with that of the discharge recorded in the 15 s period immediately prior to injection. The 15 s pre-injection period was taken as the control period. Data are expressed as either the change in the mean action potential frequency ($\Delta\bar{\chi}$; impulses s^{-1} ; Equation 1) or the change in the absolute number of action potentials evoked over the duration of the response ($\Delta\Sigma\chi$; impulses; Equation 2) according to the formula below. If no clear response was observed then the afferent activity present was measured for 15s after the injection.

$$\Delta\bar{\chi} = \bar{\chi}_{test} - \bar{\chi}_{control} \quad \text{Equation 1}$$

$$\Delta\Sigma\chi = \Sigma\chi_{test} - \Sigma\chi_{control} \quad \text{Equation 2}$$

Definitions

$\Sigma\chi$: The total number of action potentials counted in either the control (15 s) or test (duration of response) periods, $\Sigma\chi_{control}$ or $\Sigma\chi_{test}$ respectively (expressed as impulses).

$\bar{\chi}$: The frequency of action potential discharge in either the control (15 s) or test (duration of response) periods (expressed as impulses s⁻¹).

Therefore,
$$\bar{\chi}_{control} = \frac{\Sigma\chi_{control}}{t_{control}}$$

$$\bar{\chi}_{test} = \frac{\Sigma\chi_{test}}{t_{test}}$$

We are interested in the primary events at the local site of action on the afferents in the knee joint therefore delayed effects are not examined as they may possibly be the result of the injected drug circulating to additional sites to produce secondary effects on afferent discharge.

STATISTICAL ANALYSIS

Data was collected and analysed using Microsoft Excel, GraphPad Prism and GraphPad InStat software. Unpaired t-tests were used to analyse differences between the means of two normally distributed groups. When the sample size for each group was too small or the data was not normally distributed then the non-parametric Mann-Whitney U-Test was used. For paired data the Student's paired t-test

(parametric) or Wilcoxon U-Test (non-parametric) tests were used. To determine differences between the means of more than two normally distributed groups a one-way analysis of variance (ANOVA) was done and a post-hoc test (Tukey's multiple comparison) performed if the result was deemed to be significant. The medians of two or more groups of non-parametric data were analysed with a Kruskal-Wallis test and post-hoc analysis done using Dunn's multiple comparison. Correlations between normally distributed groups were determined using a Linear (Pearson) Correlation and non-parametric data were compared using a Spearman Rank Correlation. To compare the relative proportions in a population between two groups the Fisher's Exact test was used. In all cases the null-hypothesis that the variance between groups could arrive from chance was rejected at the 0.05 level. Therefore a P value of less than 0.05 was considered significant and where possible the actual P value was quoted to show proximity to the 0.05 limit.

TABLES AND FIGURES

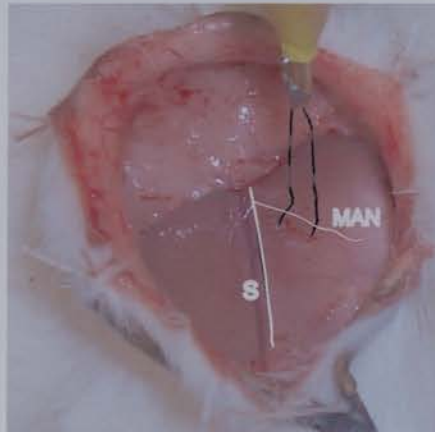
Figure 2.1 – Experimental set-up for neuronal recording from rat MAN

Photographs of the experimental set-up for recording neural activity from sensory afferents in the MAN innervating the rat knee joint. A) The left knee joint was fixed to a support and the skin tied to a small brass ring to form a cavity. The cavity was filled with heavy liquid paraffin to produce an electrically isolated system. B) Close up photograph of the MAN being laid across the bipolar platinum-iridium electrode. **S** is the saphenous nerve. For ease of viewing the saphenous and medial articular nerves have been highlighted in white.

A



B



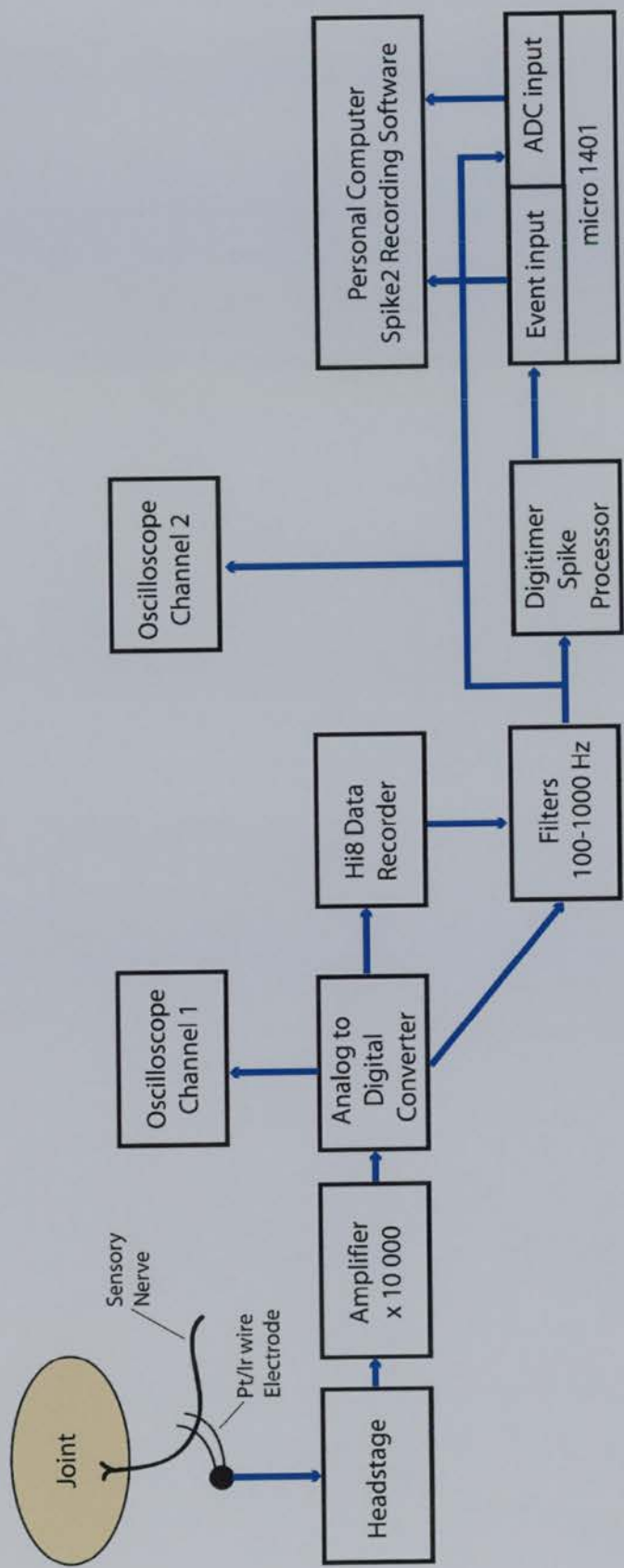


Figure 2.2 – Schematic of signal processing of neural recording
Schematic diagram of the equipment used to record, store, and analyse the neural activity from the sensory afferents innervating the rat knee joint.

3. Characterisation of nociceptors in normal and arthritic rat knee joints

3.1 INTRODUCTION

Adjuvant arthritis is one of the most well characterised models of experimental chronic inflammation. The systemic injection of bacterial cell walls or their components induces an inflammation that is thought to possess many of the characteristics seen in human rheumatoid arthritis and as such is widely used for the investigation of anti-inflammatory and anti-rheumatic drugs (Greenwald & Diamond, 1988). Polyarthritis (inflammation in multiple joints) can be produced by the injection of Freund's Complete Adjuvant (FCA) containing heat killed *Mycobacterium tuberculosis* into the footpad or tail base (Billingham, 1983; Rainsford, 1982). The inflammation produced in adjuvant arthritis is very severe and can lead to gross deformation of the joints, irreversible ankylosis, considerable discomfort, diminished weight gain or actual weight loss and spontaneous bleeding from the nose and eyes (Greenwald & Diamond, 1988). The severity of the inflammation has led some investigators to develop less invasive methods for examining arthritic joints.

A unilateral model of arthritis was previously developed in this laboratory by the sub-cutaneous injection of low doses of FCA (100-200 µg) around the ankle joint in the rat (Grubb *et al.*, 1988). The inflammation is restricted to the injected joint and allows comparison between the inflamed joint and the corresponding joint in the contralateral limb as an intra-animal control. Using this model the neural responses of articular nociceptors evoked by a number of mediators of nociception and inflammation were examined in normal and arthritic joints. The mediators investigated include 5-hydroxytryptamine (Birrell *et al.*, 1990a; Grubb *et al.*, 1988),

prostaglandins E2 and I2 (Birrell *et al.*, 1990b; Birrell *et al.*, 1991; Birrell *et al.*, 1993; Grubb *et al.*, 1991), bradykinin (Asghar *et al.*, 1997), and paracetamol and aspirin (McQueen *et al.*, 1991). Although these studies show that the model is effective for the examination of nociceptor function, it has been argued that the inflammation induced by sub-cutaneous injection of FCA is largely peri-articular and the more appropriate method of inducing arthritis was by introduction of the FCA directly into the synovial space (Butler *et al.*, 1992). Intra-articular injections into the rat ankle joint did not prove feasible, the small size and anatomical complexity meant that the injection process resulted in trauma to the joint

Previously it had been shown that it was possible to record action potential discharge from sensory afferents contained in the medial articular nerve (MAN) innervating the cat knee joint (Coggeshall *et al.*, 1983; Heppelmann *et al.*, 1986; Kanaka *et al.*, 1985; Schaible & Schmidt, 1983a; Schaible & Schmidt, 1986; Schaible & Schmidt, 1985; Schaible & Schmidt, 1983b). A method was subsequently developed in this laboratory for the induction of a unilateral arthritis in the rat knee joint by intra-articular injection of FCA and neural recordings from the MAN (Dowd, 1999). A number of groups have examined peripheral nociceptors innervating the rat knee joint (Davis & Perkins, 1993; Davis & Perkins, 1994; Davis & Perkins, 1996; McDougall *et al.*, 1994; McDougall *et al.*, 1995; Perkins *et al.*, 1995). However, these studies only examine the effect of inflammatory mediators on mechanical or thermal hyperalgesia and therefore very little is known about the pharmacological profile of chemically evoked responses from articular nociceptors.

Previous work in this laboratory has examined the evoked response of nociceptor afferents by ATP (Dowd *et al.*, 1998a; Dowd *et al.*, 1998b) and IL-1

(Kelly *et al.*, 1995a; Kelly *et al.*, 1997). The aim of this section of the studies was to examine responses evoked by known activators of peripheral nociceptors, namely capsaicin (VR₁ receptor), bradykinin (B1 and B2 receptors) and the stable ATP analog α,β -methylene-ATP (P2X₁, P2X₃ and P2X_{2/3} (heteropolymeric combination) receptors) and to determine whether these responses are altered during FCA-induced arthritis.

3.2 METHODS

AFFERENT NERVE RECORDING IN VIVO

Induction of arthritis and the recording of neural activity were carried out as described in Section 2.2. Experiments were conducted in a total of 154 male Wistar rats (body weight range 220-520 g; mean \pm sem. 374 ± 9 g). Successful neural recordings were made in 114 (74%) of the experiments and can be further divided into 76 normal and 38 arthritic animals. Arthritis was induced 14-35 days before the animals were used in electrophysiological studies. All animals displayed mild but persistent unilateral arthritis as characterised by a significant increase in the diameter of the injected joint (injected 10.7 ± 0.1 mm; uninjected 10.0 ± 0.1 mm; $n=36$; $P=0.0001$, paired t-test). Animals were anaesthetised with pentobarbitone (60 mg kg^{-1} i.p and deep anaesthesia maintained via a slow i.v. infusion at $0.5 \text{ mg kg}^{-1} \text{ min}^{-1}$ pentobarbitone) then prepared surgically as detailed in Section 2.2. Extracellular neural activity in the MAN was recorded using bipolar platinum-iridium electrodes – for detailed description of electrode placement see Section 2.2 and Figure 2.1B. Collected data were analysed off-line using Spike2 software. Drugs were

administered by close arterial injection into the right femoral cannula in a total volume of 0.1 ml.

3.3 RESULTS

AFFERENT NERVE RECORDINGS *IN VIVO*

Characterisation of afferent nerve fibres

Afferent neural discharge was recorded from 167 fibres from 76 normal knee joints and 67 fibres from 38 arthritic knee joints. Afferent fibres were characterised as either C-fibre polymodal nociceptors or A δ mechanonociceptors based on their conduction velocities, spike duration, mechanosensitivity, and their ability to be activated by capsaicin. Several low threshold mechano-sensitive A β fibres were identified during the studies, however they were not examined in detail and are not included in this thesis.

Initial experiments tried to characterise the fibres being recorded from in full using mechanical stimulation, electrical stimulation to determine conduction velocity and chemical characterisation using capsaicin, bradykinin, and $\alpha\beta$ meATP (for sample data see Table 3.1). As stated in the general introduction, a number of inflammatory and nociceptive mediators can be released anti-dromically through electrical stimulation of the nerve and by mechanical stimulation of peripheral terminals. Therefore, characterising the afferent fibres required procedures that caused the preparation in some cases to become sensitised or in other cases non-responsive. After discussions with my supervisor it was decided that the chemical

responses of the afferents should be the primary concern and therefore in the majority of experiments the determination of fibre type was based on responsiveness to the VR₁ agonist capsaicin (1-10 nmol). Fibres sensitive to capsaicin were denoted C-fibres. Fibres not responding to capsaicin but showing activation by another algogen or mechanical stimulation were denoted A δ mechanonociceptors.

When possible, to confirm capsaicin sensitive afferents as C-fibres, conduction velocities were determined using electrical stimulation. All C-fibre polymodal nociceptors tested showed slow conduction velocities ($<1.5 \text{ ms}^{-1}$) whereas A δ mechanonociceptors had a faster conduction ($> 4 \text{ ms}^{-1}$; see Table 3.1). The speed of conduction was also reflected in the duration of the action potential. Slow conducting C-fibres had significantly longer spike durations ($4.1 \pm 0.5 \text{ ms}$; $n=10$) when compared to the myelinated, faster conducting A δ mechanonociceptors ($1.3 \pm 0.1 \text{ ms}$; $n=7$; $P = 0.02$, unpaired t-test). Probing the joint capsule with a small hand-held plastic probe also activated afferent fibres.

In normal joints, of the total of 167 afferent fibres, 126/167 (75%) were classified as C-fibres and 41/167 (25%) as A δ mechanonociceptors. 61/67 (91%) afferents innervating arthritic knee joints were classified as C-fibres and 6/67 (9%) were found to be A δ mechanonociceptors.

Spontaneous discharge in nociceptors innervating normal and arthritic rat knee joints

Previous studies have shown that in adjuvant induced arthritis there is an increased proportion of spontaneous afferent discharge in the inflamed joint (Dowd, 1999; Guilbaud *et al.*, 1984). In this study spontaneous afferent firing was noted in

28/76 (37%) normal joints and in 23/38 (67%) arthritic knee joints (Figure 3.1). In joints that showed spontaneous discharge the mean action potential discharge frequency was 1.09 ± 0.55 impulses s^{-1} in normal joints and 0.87 ± 0.61 impulses s^{-1} in arthritic joints (Figure 3.2). There was no significant difference between normal and arthritic joints when comparing the frequency of the spontaneous discharge ($P = 0.43$; Mann-Whitney).

Evoked responses to known algogens capsaicin, bradykinin and $\alpha\beta$ meATP

Peripheral nociceptors innervating the rat knee joint are heterogeneous and often respond to a number of different algogens (see General Introduction; Chapter 1). In this study the afferents were characterised by determining whether there was a response to capsaicin, bradykinin, and $\alpha\beta$ meATP (Figure 3.3). Furthermore, the pooled response was analysed for the latency to onset, duration, and magnitude in terms of both the discharge frequency and the total number of action potentials evoked. The mean for each of these variables was compared in data obtained from normal and arthritic knee joints.

Capsaicin

Capsaicin (1-10 nmol, close i.a.) was used as one of the tools to distinguish between C and A δ nociceptors. However, even in fibres characterised by conduction velocity capsaicin excited all C-fibres and did not produce excitation in any of the A δ mechanonociceptors. Capsaicin (1-10 nmol, close i.a.) excited a total of 126 C-fibres and 0 A δ fibres innervating 55 normal knee joints and 61 C-fibres and 0 A δ fibres innervating 31 arthritic knee joints.

The excitation evoked by close arterial injection of capsaicin (3 nmol) was examined in detail in normal (n=32) and arthritic (n=20) knee joints (Figure 3.4). The response onset latency was 3.0 ± 0.1 s in normal knee joints and this was not significantly different in arthritic knee joints, in which delay to onset was 2.9 ± 0.2 s ($P = 0.72$; unpaired t-test; Figure 3.4A). There was also no significant difference in the duration of the evoked response to capsaicin ($P = 0.43$; Mann-Whitney) with a mean response duration of 9.2 ± 2.9 s (2.8 s) in normal joints and 4.1 ± 0.8 s (2.7 s) in arthritic knee joints (the median is shown in brackets as the duration was not normally distributed) – Figure 3.4B. Differences between normal and arthritic knee joints were seen in the magnitude of the response to capsaicin. The increase in the discharge frequency evoked by capsaicin (3 nmol) in normal knee joints (36.9 ± 3.3 impulses s^{-1}) was greater than that evoked in arthritic knee joints (26.8 ± 3.0 impulses s^{-1} ; $P = 0.04$; unpaired t-test; Figure 3.4C). Similarly, the mean total number of action potentials generated was greater in normal knee joints (295 ± 85 impulses, median: 119) when compared to inflamed knee joints (88 ± 15 impulses, median: 62; $P = 0.02$; Mann-Whitney; Figure 3.4D).

Bradykinin

Bradykinin (1-28 nmol) administration was examined in a total of 99 C-fibres and 27 A δ fibres innervating 34 normal knee joints and 37 C-fibres and 4 A δ fibres innervating 18 arthritic knee joints. Bradykinin activated 36/99 (37%) and 25/37 (68%) of C-fibres in normal and arthritic knee joints respectively; this difference was statistically significant ($P = 0.002$, Fisher's Exact test). All these fibres were also

activated by capsaicin. None of the A δ fibres, in either normal (0/27) or arthritic joints (0/4), were activated by bradykinin

Components of the excitation produced by close arterial injection of bradykinin (9 nmol) were investigated in recordings from 15 normal and 9 arthritic knee joints (Figure 3.5). There was no significant difference in the response latency between normal (6.8 ± 0.7 s) and arthritic knee joints (7.5 ± 1.5 s; $P = 0.62$; unpaired t-test; Figure 3.5A). Similarly, there was no difference in the discharge frequency with 3.6 ± 0.5 impulses s^{-1} in normal joints and 2.2 ± 0.7 impulses s^{-1} in inflamed joints ($P = 0.15$; unpaired t-test; Figure 3.5C). The duration of the response was significantly increased in arthritic animals when compared to normal animals (153.6 ± 24.5 s and 35.4 ± 6.4 s respectively; $P = 0.0001$, unpaired t-test; Figure 3.5B). As the discharge frequency was similar between normal and arthritic animals the change in duration meant that the total number of action potentials generated was also significantly increased in arthritic knee joints (normal: 129 ± 35 impulses, arthritic: 284 ± 77 impulses; $P = 0.048$; unpaired t-test; Figure 3.5D)

α,β – methylene ATP

$\alpha\beta$ meATP (19 – 190 nmol) elicited a fast onset, high frequency response from a total of 11/80 (14%) of C-fibres and 28/28 (100%) of A δ fibres in normal joints (n=38) and 4/33 (12%) of C-fibres and 7/7 (100%) of A δ fibres in arthritic joints (n=17). There was no significant difference between the proportion of either C-fibre or A-fibre afferents ($P = 0.77$ and $P = 1.23$ respectively; Fisher's Exact test) excited in normal and arthritic knee joints. All the C-fibres activated by $\alpha\beta$ meATP were also activated by capsaicin in both normal and arthritic joints and bradykinin

activated 3/11 (27%) of $\alpha\beta$ meATP sensitive C-fibres in normal joints and 1/4 (25%) in arthritic joints; this was not significant ($P = 1.00$, Fisher's Exact test). The A δ mechanonociceptors activated by $\alpha\beta$ meATP were not activated by either capsaicin or bradykinin.

Close arterial injection of $\alpha\beta$ meATP (60 nmol) elicited an excitation of rapid onset, and short duration that was similar in normal and arthritic knee joints (Figure 3.6). There were no significant differences in the mean response latency (normal: 2.6 ± 0.3 s, arthritic: 2.8 ± 0.1 s; $P = 0.56$; unpaired t-test), the mean duration of the response (normal: 1.6 ± 0.2 s, arthritic: 1.5 ± 0.5 s; $P = 0.89$; unpaired t-test), the mean discharge frequency (normal: 9.0 ± 2.4 impulses s^{-1} , arthritic: 9.6 ± 2.0 impulses s^{-1} ; $P = 0.88$; unpaired t-test), or the mean number of action potentials evoked (normal: 12 ± 3 , arthritic: 17 ± 9 ; $P = 0.56$; unpaired t-test).

3.4 DISCUSSION

This section of the thesis examined changes in both spontaneous and chemically evoked primary afferent discharge in arthritic rat knee joints and compared these with responses from normal joints. The MAN in the cat knee joint has been shown to consist primarily of afferents arising from the joint capsule of which approximately 80% were unmyelinated C-fibres and 20% were myelinated fibres of which the majority were A δ fibres (Heppelmann *et al.*, 1988; Langford & Schmidt, 1983). The innervation of the rat knee joint has also been examined, however much of the work focuses on the posterior articular artery (PAN) and not the MAN (Hildebrand *et al.*, 1991). What is known of the innervation of the MAN into the rat knee joint is that approximately 1/3 of the fibres present in the bundle are

afferents (Hildebrand *et al.*, 1991). To ensure that we were recording only afferent activity the saphenous nerve was cut centrally. Assuming that the proportion of afferents being recorded is similar to the overall proportion of afferents in the nerve bundle, the results of this study for the proportion of myelinated to unmyelinated fibres was consistent with those seen in the cat with 75% of recorded afferents being classified as C-fibres and 25% classified as A δ fibres. Furthermore, this was also consistent with earlier findings from this laboratory in the rat knee joint (Dowd, 1999).

As efferent activity was not being recorded, all C-fibres were deemed to be nociceptors as their firing was induced by capsaicin, which is known to produce pain in both animals and humans (Szallasi & Blumberg, 1999). It was not possible to characterise specifically the A δ fibres into either mechanoreceptors or high threshold mechanonociceptors, as it was not technically feasible to move the joint to produce noxious mechanical stimulation, such as over rotation of the joint, as is used in other experimental models (Heppelmann & Pawlak, 1997a; Heppelmann & Pawlak, 1999). In addition the excessive use of direct mechanical stimulation of the joint causes desensitisation in our preparation and as such when tested, mechanical thresholds were left until the end of an experiment.

Determination of A-fibre type was not permitted by noxious mechanical stimulus. However, it has previously been shown that although A δ mechanonociceptors are insensitive to excitation by capsaicin under normal conditions, A δ mechanoreceptors, for non-nociceptive sensory input and proprioception, do respond to capsaicin after a long latency (Szolcsanyi, 1987). As

this was not seen in any of the myelinated fibres we recorded from, it is assumed that all fibres tested were transmitting nociceptive information.

Although the proportion of C to A δ fibres was consistent with that seen in the cat and previously shown in the rat in normal joints, there was a difference in the relative number of each fibre in the arthritic knee joints of rats. In the arthritic joint the vast majority of fibres recorded from were C-fibre polymodal nociceptors (91%) with only a small number of A δ mechanonociceptors (9%). The first explanation of this finding is simply a skew in the sampling of afferents in these arthritic joints. It would be very interesting for future work to examine changes in number and fibre type of afferents in the MAN of chronically inflamed joints. However, recent reports point to the fact that primary sensory afferents may change under conditions of neuropathy or chronic inflammation producing alterations in their central projections into the dorsal horn. In addition, neuropathy and inflammation are capable of inducing the production of neuropeptides in medium and large diameter sensory neurons (A δ and A β myelinated fibres respectively). This has been shown in the rat DRG after sciatic nerve lesion and FCA induced inflammation with the production of brain-derived neurotrophic factor (BDNF) (Zhou *et al.*, 1999), and calcitonin gene related peptide (CGRP) (Bulling *et al.*, 2001; Ohtori *et al.*, 2001), and in sensory nerves innervating the guinea pig airway, where exposure to NGF produced expression of substance P (Hunter *et al.*, 2000). In combination, these results show that under severe conditions such as chronic inflammation, it is possible for the A δ fibres to change their phenotype causing A δ mechanonociceptors, normally insensitive to capsaicin, to become C-fibre-like nociceptors capable of being activated by capsaicin. Recent reports show that in response to partial nerve injury

the level of VR₁ receptor mRNA increases in the cell bodies of A-fibres in the rat DRG (Hudson *et al.*, 2001). Since one of the criteria for classification of A δ fibres is insensitivity to capsaicin, it would be necessary to determine under conditions of chronic inflammation whether A-fibres will begin to express the VR₁ receptor, and also whether changes in VR₁ receptor expression in the cell body equates to receptor expression at the peripheral terminal. If this were the case it would begin to explain the apparent lack of A δ fibres in the arthritic joint in our model. Further work will be required to isolate myelinated fibres using conduction velocities to determine whether in our model the induction of chronic arthritis is capable of directing A-fibres to become activated by stimuli that previously only activated C-fibre polymodal nociceptors.

It has been previously been shown in the rat ankle joint (Asghar *et al.*, 1997; Guilbaud *et al.*, 1985b) and the cat knee joint (Schaible & Schmidt, 1985) that in chronic inflammation induced by FCA there is an increase in both the number of afferents showing spontaneous activity and, in addition, those afferents that are spontaneously active produced higher action potential discharge frequencies than that seen in normal joints. The present study in the rat knee joint does show a significantly higher proportion of afferent fibres with spontaneous firing in arthritic joints, however, there was no significant difference between normal and arthritic joints in terms of the frequency of the discharge.

If it is assumed that signalling from peripheral sensory afferents provides continual information on both the external and internal environment to the central nervous system, then changes in any of the features of the response may be capable of changing the perception of the input centrally. For this reason future experiments

will need to examine, in more detail, different components of the afferent responses generated and look for differences between normal and arthritic joints. Using recent developments in processing software, it will be possible to examine the nature of both the spontaneous and evoked response to determine changes in the coding of the sensory information. For example, in addition to the absolute discharge or the frequency it will be possible to examine the grouping of the spikes and the inter-spike interval to determine if these are altered in chronically inflamed joints.

As there was no alteration in spontaneous discharge frequency recorded from afferents innervating normal and arthritic joints, changes in the evoked responses to known algogens (including capsaicin, bradykinin and $\alpha\beta\text{meATP}$) were studied to determine whether their response characteristics were altered under conditions of chronic inflammation.

ATP and capsaicin activate ligand-gated ion channels, namely the purinoceptor (P2X) and the vanilloid receptor (VR₁) respectively. It was confirmed that $\alpha\beta\text{meATP}$ does activate sensory afferents in the rat knee joint (Dowd *et al.*, 1997) presumably through its selective activation of P2X₁, P2X₃ and/or P2X_{2/3} receptors. The localisation of the P2X₃ receptor to small diameter sensory neurons in the DRG (Novakovic *et al.*, 1999; Rae *et al.*, 1998) and to the peripheral terminals of these afferents (Vulchanova *et al.*, 1997) indicates that it is most likely the receptor being activated. There was however no significant difference in the excitation evoked by $\alpha\beta\text{meATP}$ in afferents innervating normal and arthritic joints – meaning that chronic inflammation did not sensitise the afferents to stimulation by $\alpha\beta\text{meATP}$. This result conflicts with recent reports showing that currents mediated by the P2X₃ receptor were potentiated by substance P and bradykinin (Paukert *et al.*, 2001).

Furthermore, following FCA-induced peripheral inflammation, recordings from dissociated rat DRG neurons showed a threefold increase in P2X₃ mediated ATP currents and the expression of P2X₃ receptor was significantly increased (Xu & Huang, 2002).

The response evoked by capsaicin was significantly different when comparing afferents innervating normal and arthritic joints, however in the chronically inflamed joints the excitation evoked was less than that seen in normal animals, implying a desensitisation of the afferents. There is no information of the expression of the VR₁ receptor following chronic inflammation and furthermore recent reports are conflicted in the expression and sensitivity of the VR₁ receptor following acute (48 hours) inflammation. In the rat DRG it has been shown that FCA-induced peripheral inflammation did not alter the level of VR₁ expression (Sanchez *et al.*, 2001). However, work by Carlton and Coggeshall (2001) suggests that peripheral sensitisation following inflammation is a result of an increase in the number of VR₁ receptors present at the peripheral terminal. One potential mechanism for an increase in receptor levels comes from the fact that activation of phospholipase C (PLC) by nerve growth factor (NGF) or bradykinin triggers expression of VR₁ (Chuang *et al.*, 2001).

Although chronic inflammation did not appear to sensitise the ion-channel receptors, bradykinin, acting through a G-protein coupled receptor, did show a significantly greater excitation in afferents innervating the arthritic joint. Both the duration of the response and the absolute number of impulses generated in the arthritic rat joint were increased when compared to normal joints. One potential explanation for this finding lies in the fact that in chronically inflamed joints the pH

of the tissue is lower than usual (Wall & Melzack, 1999). Under these conditions the enzymes responsible for the breakdown and elimination of bradykinin are likely inhibited (Edery & Lewis, 1962) leaving the molecule free to act for a longer time. Similarly, it has been shown that bradykinin modulates ionic channels particularly at low pH to prolong activation in isolated, small diameter, rat DRG neurons (Kress *et al.*, 1997).

Another possible explanation involves the activation of both of the bradykinin receptors. Bradykinin acts on two receptors, B1 and B2. The B2 receptor is constitutively expressed and the B1 receptor is inducible and shows a significant increase in expression following tissue injury or inflammation (for reviews see Calixto *et al.*, 2000; Couture *et al.*, 2001). It is possible that in normal animals bradykinin is acting on the B2 receptor to produce its effect and then during chronic inflammation the B1 receptor is also expressed at high levels producing a combined response. The hypothesis that B2 may be involved in acute pain and B1 in the more chronic phase was strengthened by the report that chronic constriction injury caused an upregulation of both receptors in the rat lumbar DRG (Ma *et al.*, 2000) and work from this laboratory showing excitation by the B1 agonist desArg⁹-bradykinin in afferents innervating arthritic but not normal rat knee joints (Kelly, 1998). Furthermore, following chronic constriction injury in the rat, B2 antagonists were able to produce anti-nociception at all time points whereas the B1 antagonist was only effective after 14 days, therefore in a more chronic phase (Levy & Zochodne, 2000).

Further work using specific agonists and antagonists for the two bradykinin receptors would determine whether the increased response to bradykinin during

chronic inflammation is due to the presence of B1 receptors. Although it is known that levels of B1 receptor are greatly increased during inflammation there has been recent debate in the literature as to whether or not the B1 receptor is constitutively expressed in sensory afferents. Several studies showed immunohistological evidence for its presence in small diameter, peptidergic neurons in the DRG (Ma, 2001; Ma *et al.*, 2000; Wotherspoon & Winter, 2000). Others were not able to elicit a B1 agonist dependent excitation from peripheral nociceptors in the rat knee (Kelly, 1998) or find B1 mRNA in rat DRG (Brand *et al.*, 2001). Probably the most conclusive proof of constitutive B1 receptor expression comes from the finding that mice lacking the receptor display hypoalgesia under normal conditions (Pesquero *et al.*, 2000).

The further understanding of differential response patterns and chemosensitivity of sensory afferents innervating arthritic joints could lead to new targets for drugs specific to a chronic inflammatory condition such as rheumatoid arthritis.

TABLES AND FIGURES

Fibre Type	Mechanically Sensitive	Conduction velocity (m/s)	Spike Duration (ms)	Capsaicin Sensitive	Bradykinin Sensitive	ATP or $\alpha\beta$ meATP Sensitive
A δ	x	5.3	1.2			
A δ		5.8	1.3			x
A δ	x	3.1	1.6			
A δ		3.4	1.1			x
A δ	x	3.6	0.9			
A δ	x	5.6	1.5			x
A δ	x	3.2	1.6			x
C	x	0.4	6.1	x		
C	x	0.6	3.0	x	x	
C		0.5	7.5	x		x
C		0.7	2.6	x		x
C		0.7	3.0	x		
C	x	1.2	3.6	x		
C	x	1.1	2.9	x		
C	x	0.5	5.6	x		
C	x	0.8	5.0	x	x	
C	x	1.7	3.2	x		

Table 3.1 – Characteristics of nociceptors innervating the normal rat knee joint

Sample table of data collected to characterise A δ and C fibres innervating the rat knee joint. A δ fibres had conduction velocities of $4.3 \pm 0.5 \text{ m s}^{-1}$ (n=7) and spike durations of $1.3 \pm 0.1 \text{ ms}$ (n=7). None of the A δ fibres were sensitive to capsaicin but some were sensitive to ATP or its stable analogue $\alpha\beta$ meATP. C fibres had conduction velocities of $0.8 \pm 0.1 \text{ m s}^{-1}$ (n=10) and spike durations of $4.1 \pm 0.5 \text{ ms}$ (n=10). C fibres showed responses to capsaicin, bradykinin and ATP or $\alpha\beta$ meATP. It was not always possible to determine if the fibre could be mechanically stimulated due to restricted access to the joint surface.

Figure 3.1 – Proportion of afferent nerves showing spontaneous firing in recordings from normal and arthritic rat knee joints

Proportion of afferent fibres contained in a portion of the MAN that showed spontaneous activity in recordings made from A) normal and B) arthritic rat knee joints. Fibres characterised as non-active (silent) showed no spontaneous (on-going) basal discharge, but were activated by either direct chemical or mechanical stimulation.

A greater proportion of afferents showed spontaneous activity in arthritic joints in comparison with normal joints ($P=0.03$; Fisher's exact test).

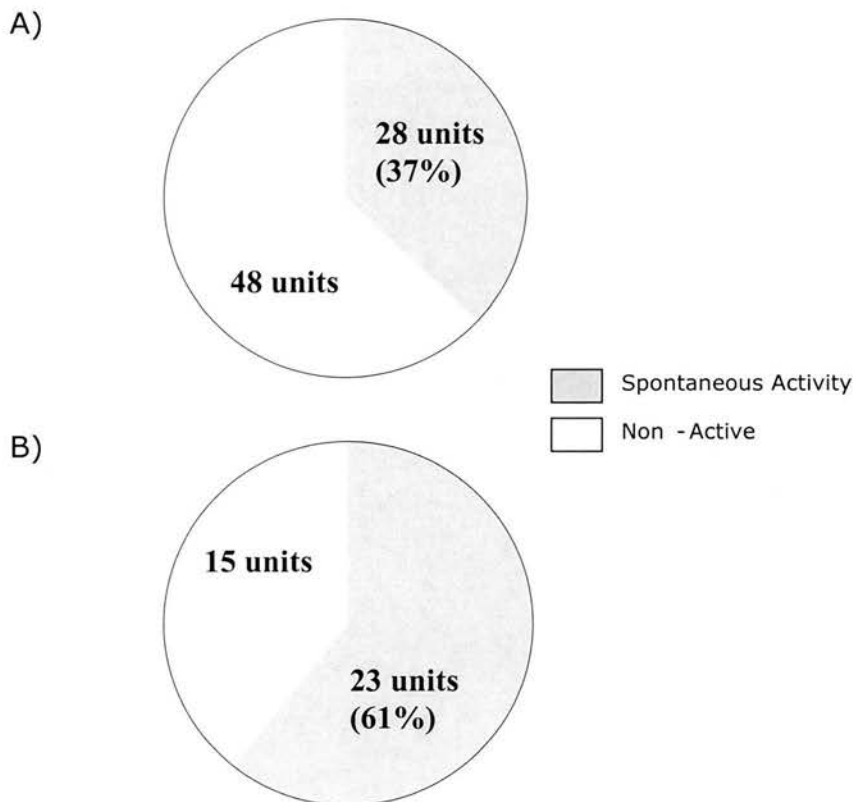


Figure 3.2 – Basal spontaneous activity in fibres of the MAN in normal and arthritic rat knee joints

The action potential discharge frequency (impulses s^{-1}) of spontaneously active fibres recorded from a portion of the MAN innervating normal and arthritic rat knee joints. Data shown represents the mean basal discharge \pm sem for responses from normal ($n=28$) and chronically arthritic ($n=23$) joints.

There was no significant difference in the frequency of the spontaneous discharge between normal and arthritic joints.

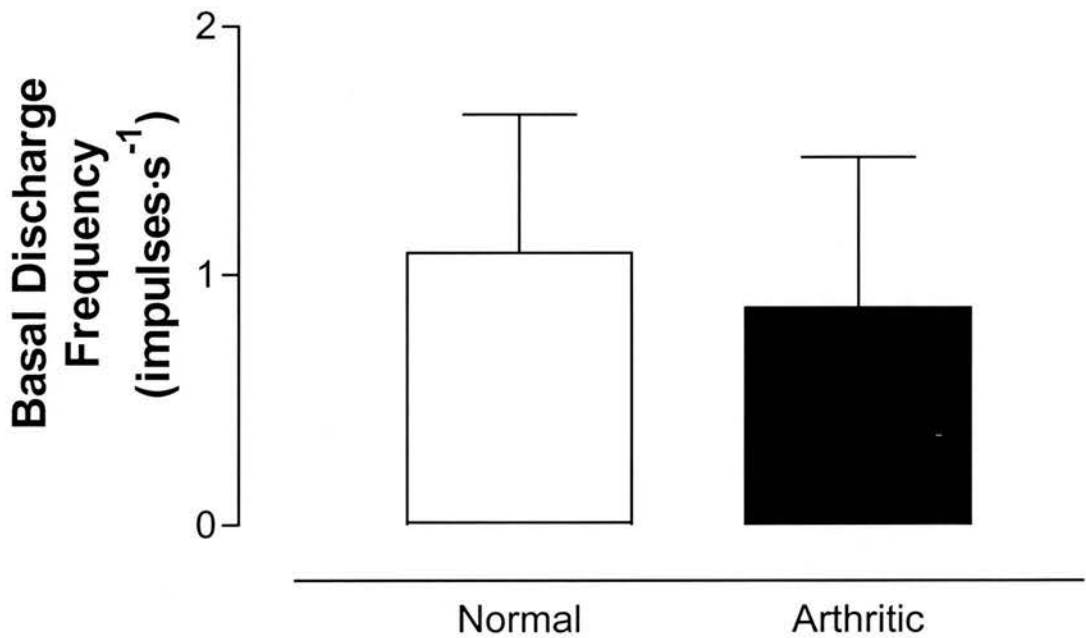


Figure 3.3 – Typical responses evoked by capsaicin, bradykinin and ATP in a multi-fibre recording from the MAN in a normal rat knee joint

Multi-fibre neural discharge (left panel) showing the evoked response from a portion of the MAN innervating a normal rat knee joint by a close arterial injection of A) capsaicin (9 nmol, activated fibres 1, 3-6), B) bradykinin (9 nmol, activated fibres 3 and 4), and C) $\alpha\beta$ meATP (60 nmol, activated fibres 2 and 6). Filters were set to allow only discharge above noise level to be captured. Individual fibres are shown on the right panel

Typical responses showing high frequency, transient responses for the ion channels gated by receptors for capsaicin and $\alpha\beta$ meATP and the longer-lasting response produced by bradykinin acting through a G-protein coupled receptor.

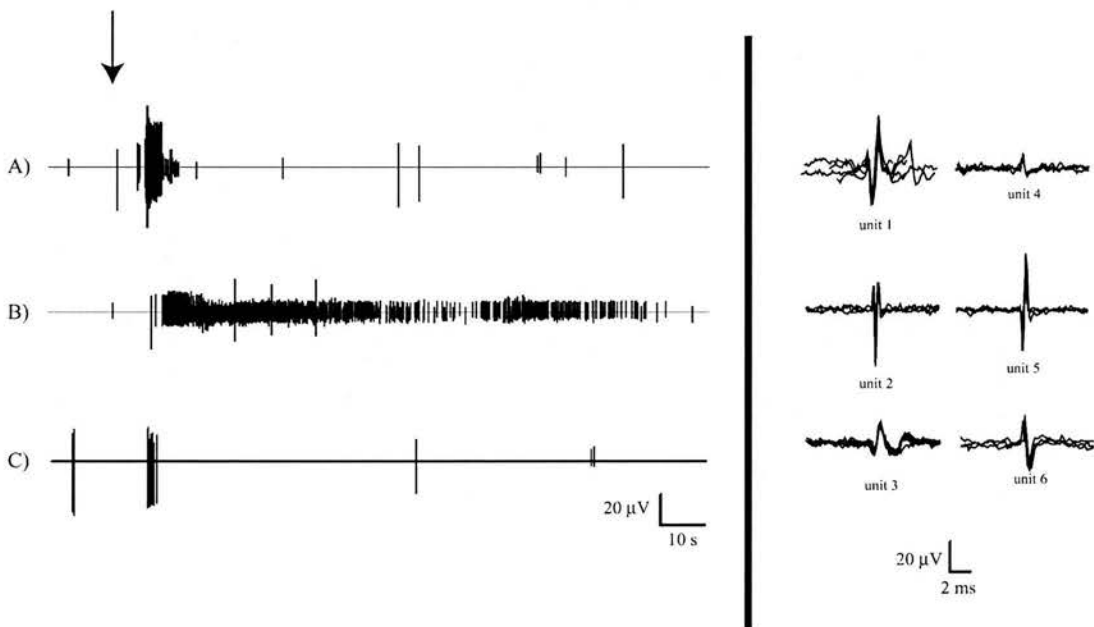


Figure 3.4 – Comparison of the capsaicin-evoked response obtained from afferent nerves innervating normal and arthritic rat knee joints.

Comparison of the A) latency to onset, B) response duration, C) change in action potential discharge frequency and D) change in total action potential discharge following a close arterial injection of capsaicin (9 nmol). Data shown represents the mean \pm sem for pooled responses from normal (n=32) and arthritic (n=20) joints. * P<0.05.

There was a significant decrease in the magnitude of the response in arthritic joints when compared to normal joints.

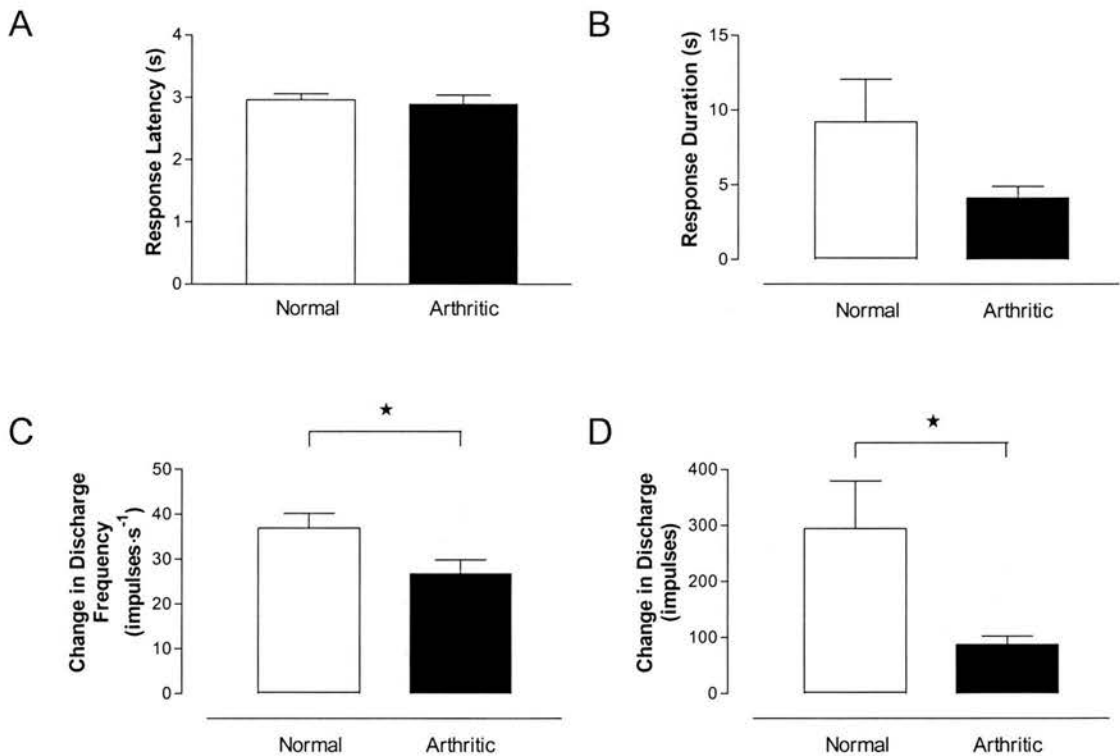


Figure 3.5 – Comparison of the bradykinin-evoked response obtained from afferent nerves innervating normal and arthritic rat knee joints.

Comparison of the A) latency to onset, B) response duration, C) change in action potential discharge frequency and D) change in total action potential discharge following a close arterial injection of bradykinin (9 nmol). Data shown represents the mean \pm sem for pooled responses from normal (n=15) and chronically arthritic (n=9) joints. *** $P < 0.001$, * $P < 0.05$.

There was a significant increase in the duration of the response in arthritic joints when compared to normal joints. This increase in duration resulted in an overall increase in the number of action potentials generated.

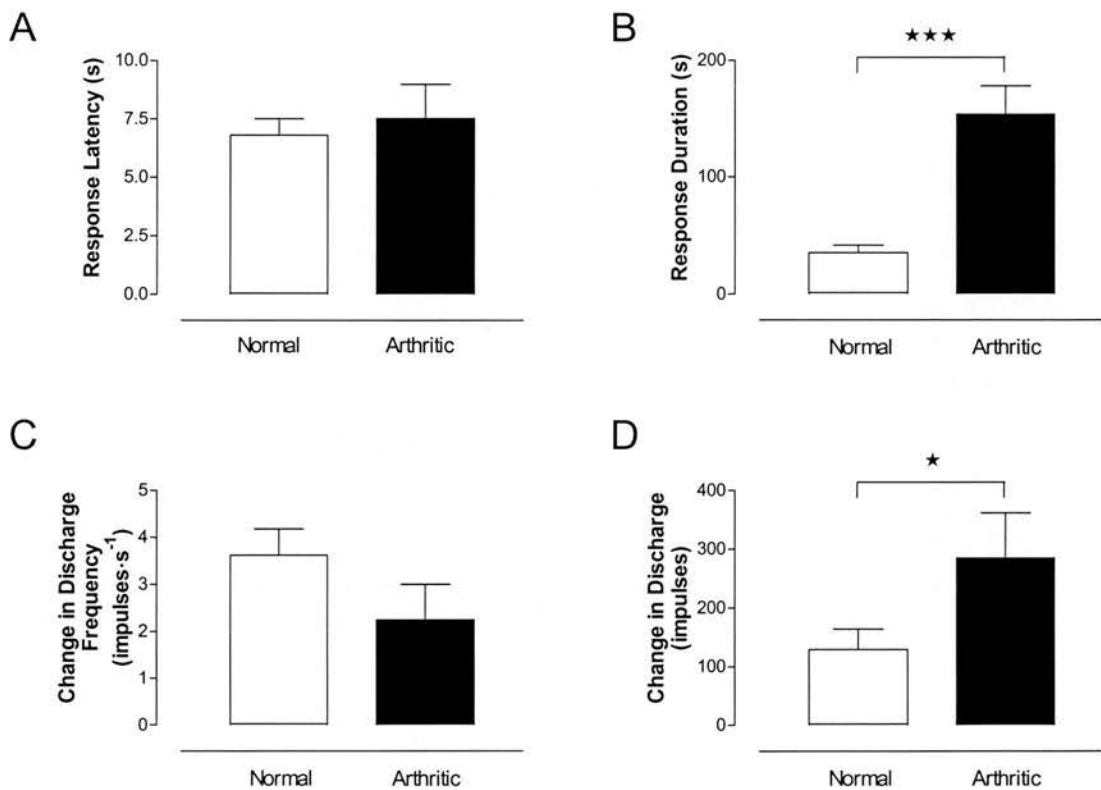
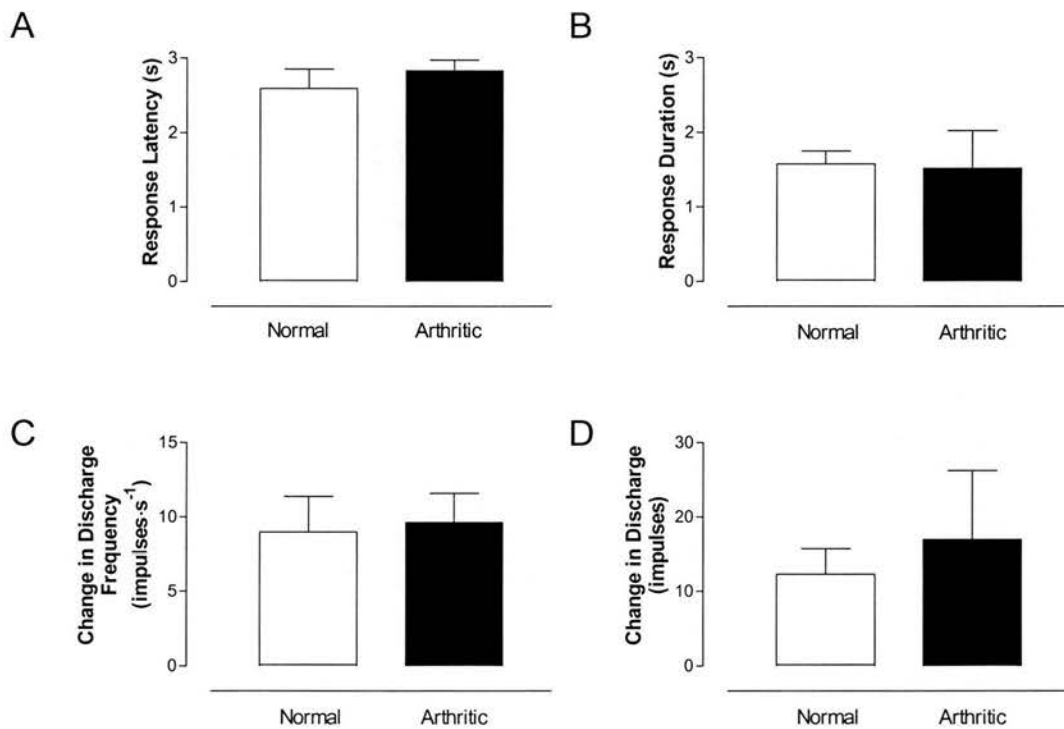


Figure 3.6 – Comparison of the $\alpha\beta$ meATP-evoked response obtained from afferent nerves innervating normal and arthritic rat knee joints.

Comparison of the A) latency to onset, B) response duration, C) change in action potential discharge frequency and D) change in total action potential discharge following a close arterial injection of $\alpha\beta$ meATP (60 nmol). Data shown represents the mean \pm sem for pooled responses from normal (n=13) and chronically arthritic (n=6) joints.

There was no significant difference in any of the elements of the response evoked by $\alpha\beta$ meATP between normal and arthritic joints.



4. Effect of somatostatin on knee joint nociceptors

4.1 INTRODUCTION

Somatostatin (SOM) is a neuropeptide that is widely distributed throughout the body. It was first discovered in 1968 as a hypothalamic extract that had an endocrine function in its ability to inhibit the release of growth hormone from the pituitary (Krulich *et al.*, 1968). It has since been shown that in addition to its endocrine role SOM displays a wide variety of effects both centrally and peripherally, including acting as a neuromodulator and as a mediator of inflammation, with the vast majority of its actions being inhibitory (for reviews see Reichlin, 1983; Reichlin, 1995 and Schindler *et al.*, 1996).

The peptide occurs naturally in two forms in mammals, a fourteen amino acid peptide (SOM-14) and a twenty-eight amino acid peptide (SOM-28). Both isoforms are derived from a precursor protein preprosomatostatin and they become active once cleaved from the parent protein, which occurs in response to inflammatory mediators such as LPS, IFN- γ , TNF- α , and prostaglandin E2 (Elliott *et al.*, 1998). The diverse effects of SOM are mediated by five specific somatostatin receptors (SSTR₁₋₅), which were identified by molecular cloning. The receptors can be divided into two classes according to their pharmacological characteristics. The first class (SSTR₂, SSTR₃ and SSTR₅) exhibits a high affinity for smaller peptide analogs of somatostatin, including octreotide, and a second class (SSTR₁ and SSTR₄) that do not (Schindler *et al.*, 1996). The SSTR₂ receptor has also been further subdivided into SSTR_{2A} and SSTR_{2B} as splice variants differ in the intracellular C-terminal, and therefore may be capable of activating alternative signal pathways. All the receptors are closely related, and all produce their effects through G-protein coupled

mechanisms (reviewed in Patel, 1997; Patel, 1999). SOM-14 and SOM-28 show equal activity at SST₁₋₄, however the SST₅ receptor shows a 10-15-fold selectivity for SOM-28 over SOM-14. *In vivo* the peptides have a short half-life of 1-3 minutes and are rapidly degraded by tissue and serum peptidases, therefore to facilitate studies a number of long lasting synthetic analogs have been developed, including octreotide (Sandostatin) that increases the half-life to 1.5-2 hours and prolongs the duration of action to approximately 12 hours (Brown, 1990). Octreotide shows highest affinity for the SSTR₂ and SSTR₅ receptors. The amino acid structures of SOM-14, SOM-28, and octreotide are shown in Figure 4.1.

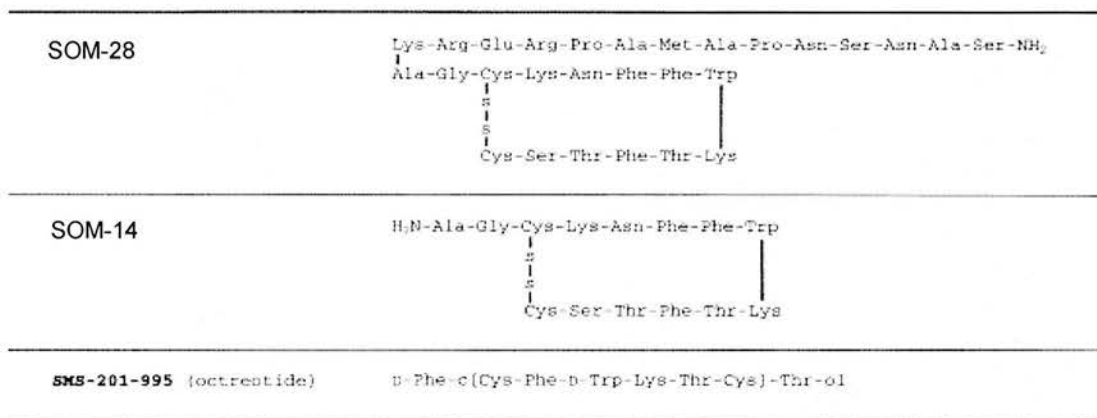


Figure 4.1 – Structure of somatostatin and synthetic analog octreotide

Amino acid structure of the two naturally occurring somatostatin analogs, somatostatin-28 (SOM-28) and somatostatin-14 (SOM-14) and the long-lasting analog octreotide (Sandostatin). Adapted from Schindler et al. (1996).

SOM has been shown to possess both anti-inflammatory and anti-nociceptive activity. Its anti-inflammatory properties have been demonstrated in animal models of both acute and chronic inflammation (Corsi *et al.*, 1997; Karalis *et al.*, 1994;

Matucci-Cerinic *et al.*, 1995) and in human chronic inflammatory conditions such as osteo- and rheumatoid arthritis (Ciocci *et al.*, 1994; Coari *et al.*, 1995; Fioravanti *et al.*, 1993; Fioravanti *et al.*, 1995; Russo *et al.*, 1997; Silveri *et al.*, 1994). SOM is released from many immune cells including macrophages and T-cells in response to cytokines or other inflammatory mediators (Bjorling *et al.*, 1997; Elliott *et al.*, 1998). Once released, activation of the SSTR_{2A} receptor, also present on the majority of immune cells, inhibits the release of other pro-inflammatory mediators including INF- γ (Elliott *et al.*, 1999), TNF- α , IL-1 β , and IL-6 (Grimaldi *et al.*, 1997; Peluso *et al.*, 1996). Furthermore, there is a substance P (SP)/SOM immunoregulatory circuit where increased levels of one neuropeptide inhibits the expression of the other (Blum *et al.*, 1998; Campbell & Scanes, 1995). SOM and SP have opposite effects on different aspects of the inflammatory response such as neutrophil migration (Partsch & Matucci-Cerinic, 1992) and activation (Kolasinski *et al.*, 1992) and the production of metalloproteinases in RA (Sakane & Suzuki, 1998). In addition to being present in immune cells, SOM is also produced in peripheral sensory neurons and is released from the peripheral terminals where it can act locally on adjacent terminals or immune cells (Hokfelt *et al.*, 2000; Szolcsányi *et al.*, 1998a), and is also capable of acting systemically (Szolcsányi *et al.*, 1998b). Under conditions of chronic inflammation the levels of SOM and its receptors are increased. In human RA patients it has been shown that not only is there an increase in SOM levels in the synovium of arthritic joints but there is also a corresponding increase in the level of the SSTR_{2A} receptor which becomes localised to the synovium, suggesting a prominent role in the condition (ten Bokum *et al.*, 1999; van Hagen *et al.*, 1999).

In addition to producing anti-inflammatory effects, both animal and human studies have shown that SOM is also capable of causing analgesia. Presumably, one mechanism for SOM-mediated analgesia is simply through its action to reduce inflammation, and thereby reducing nociceptor sensitisation/activity by inflammatory mediators. However it has been shown that SOM also acts directly on sensory afferents to inhibit nociception (Heppelmann & Pawlak, 1999). Anatomical studies of the rat DRG show the presence of both SSTR₂ splice variants (Schulz *et al.*, 1998; Selmer *et al.*, 2000) and the SSTR₃ receptor (Senaris *et al.*, 1995). Targeting these receptors on peripheral afferents using SOM or its analogs inhibited activation of mechanonociceptors in both the normal and the inflamed knee joint of the rat (Heppelmann & Pawlak, 1997a). Furthermore, somatostatin is involved in the control of normal tissue and joint homeostasis as there is tonic expression of somatostatin, as evidenced by treatment with the specific antagonist cyclo-somatostatin, which was able to increase the sensitivity of articular mechanonociceptors to noxious movements (Heppelmann & Pawlak, 1999).

The present investigation used electrophysiological methods to examine the hypothesis that SOM, or its long lasting analog octreotide, will inhibit the function of polymodal nociceptors innervating the rat knee joint. It has already been shown that somatostatin can decrease mechanonociceptor function in rat articular afferents, so the present study examined the effect of SOM or octreotide on the spontaneous and chemically evoked discharge from C-fibre polymodal nociceptors innervating both normal and arthritic rat knee joints.

4.2 METHODS

AFFERENT NERVE RECORDING *IN VIVO*

Induction of arthritis and subsequent neural recordings from afferent nerves innervating the knee joint were carried out as described in Section 2.2. Experiments were conducted in a total of 23 normal and 6 arthritic Wistar rats (body weight range 220-520 g; mean \pm sem. 372 ± 14 g). Arthritis was induced 14-35 days before the animals were used in electrophysiological studies. Animals displayed mild but persistent unilateral arthritis as characterised by a significant increase in the diameter of the injected joint (injected 11.0 ± 0.2 mm; uninjected 10.2 ± 0.2 mm; $n=6$; $P=0.008$, paired t-test). Animals were anaesthetised with pentobarbitone (60 mg kg^{-1} i.p and deep anaesthesia maintained via a slow i.v. infusion at $0.5 \text{ mg kg}^{-1} \text{ min}^{-1}$) then prepared surgically as detailed in Chapter 2.2. Extracellular neural activity in the MAN was recorded using bipolar platinum-iridium electrodes. Collected data was analysed off-line using Spike2 software. Drugs were administered by close arterial injection into the right femoral cannula in a total volume of 0.1 ml. For the majority of experiments the stable analog octreotide was used due to the rapid metabolism of SOM-14 *in vivo*.

4.3 RESULTS

CHARACTERISATION OF AFFERENT NERVE FIBRES

Afferent neural discharge was recorded from a total of 52 individual fibres from 23 normal knee joints and 11 individual fibres innervating 6 arthritic knee joints. The afferents were classified as either C-fibre polymodal nociceptors or A δ

mechanonociceptors based on their conduction velocities, spike duration, mechanosensitivity and excitation by capsaicin, as described in Chapter 2.2. In normal joints, 43/52 (83%) of fibres were C-fibre polymodal nociceptors and 9/52 (17%) were A δ mechanonociceptors. In arthritic joints, 10/11 (91%) of afferents were C-fibres and 1/11 (9%) was an A δ fibre. There was no significant difference in the proportion of C-fibres or A δ -fibres ($P = 0.68$ and $P = 0.68$ respectively, Fisher's exact test) innervating normal and arthritic joints. Spontaneous discharge in these fibres was present in 9/52 fibres (17%) in normal joints, with a mean frequency of 0.47 ± 0.31 impulses s^{-1} , and in arthritic joints 6/11 (55%) afferents showed spontaneous activity with a mean frequency of 0.31 ± 0.07 impulses s^{-1} . There was a significant increase in the number of afferents showing spontaneous discharge in nociceptors innervating arthritic joints ($P = 0.02$, Fisher's exact test), however there was no statistical difference in the mean discharge frequency ($P = 0.67$; unpaired t -test) in recordings from normal and arthritic knee joints.

NEITHER OCTREOTIDE NOR SOMATOSTATIN-14 EXCITED PERIPHERAL NOCICEPTORS

The reported inhibitory nature of somatostatin would lead one to expect that somatostatin would not directly activate peripheral nociceptors. However, one report has shown that SOM causes excitation of spinal neurons purportedly via C-fibre activation (Wiesenfeld-Hallin, 1985).

A single close-arterial injection of octreotide (1-98 nmol) did not excite C-fibre polymodal nociceptors (0/36; 0%) or A δ mechanonociceptors in normal joints. Similar results were found in arthritic joints: no C-fibres (0/8 ;0%) or A δ fibres (0/4;

0%) were activated. As octreotide is only an agonist at SSTR₂ and SSTR₅ receptors, the native protein SOM-14 was also tested in a few animals. The same results were obtained: SOM-14 (0.6-60 nmol) was inactive in afferents innervating normal (C-fibres: 0/7; 0% activated, and A δ fibres: 0/1; 0% activated) and arthritic joints (C-fibres: 0/2; 0% activated, and A δ fibres: 0/1; 0% activated).

EFFECT OF OCTREOTIDE ON SPONTANEOUS AFFERENT DISCHARGE FROM NORMAL AND ARTHRITIC KNEE JOINTS

In recordings that showed spontaneous afferent discharge we examined the change in action potential discharge frequency for 60 s following the injection of octreotide (10 nmol i.a.; Figure 4.2). In normal joints there was no significant change in the afferent discharge frequency of 2.19 ± 0.73 impulses s⁻¹ before and 2.57 ± 0.99 impulses s⁻¹ after close arterial injection of octreotide ($P = 0.28$; paired t-test; $n=5$ for both; Figure 4.2A). The same response was seen in arthritic joints with a frequency of 2.51 ± 0.53 impulses s⁻¹ before and 2.72 ± 1.12 following octreotide ($P = 0.85$; paired t-test; $n=3$ Figure 4.2B). The afferent discharge was also measured 5 minutes following the injection of octreotide, and again there was no change in the frequency (data not shown).

Single experiments in a normal and an arthritic animal were also undertaken using higher doses of octreotide (29 and 98 nmol) and somatostatin-14 (6 – 60 nmol), but again there was no change in spontaneous discharge up to 5 min after drug administration.

EFFECT OF OCTREOTIDE ON RESPONSES FROM KNEE JOINT AFFERENTS EVOKED BY CAPSAICIN, BRADYKININ AND $\alpha\beta$ meATP

The ability of octreotide to act as a neuromodulator was examined by investigating excitation evoked by known algogens before and after the injection of the neuropeptide. Responses to capsaicin, bradykinin and $\alpha\beta$ meATP were measured before, and 5 minutes following, close arterial injection of octreotide (10 nmol; Figure 4.2). A minimum of 20 minutes was allowed between successive injections of algogen in order to minimise desensitisation.

Capsaicin

The excitation of knee joint afferents evoked by capsaicin (3 nmol) before and after octreotide (10 nmol) was examined in a total of 12 afferent fibres from 5 normal knee joints (Figure 4.3A). The mean action potential discharge frequency evoked by caps before the injection of octreotide was 27.5 ± 6.2 impulses s^{-1} and the mean number of action potentials generated was 60 ± 8 impulses. Five minutes after the administration of octreotide the response to the same dose of capsaicin averaged 39.5 ± 12.2 impulses s^{-1} and the mean number of action potentials was 83 ± 22 impulses. There was no significant difference in the response obtained before and after octreotide, either in terms of the mean discharge frequency ($P = 0.29$; paired t-test), or the mean total number of potentials evoked ($P = 0.25$; paired t-test).

The native peptide was also tested to see whether it could modulate the response to capsaicin (3 nmol). SOM-14 (6 nmol) in 3 fibres from 2 normal knee joints had no significant effect on capsaicin-evoked discharge frequency: mean responses to capsaicin were 24.9 ± 14.8 impulses s^{-1} before, and 23.9 ± 5.8 after

SOM-14. Therefore, neither the peptide, nor the synthetic analog octreotide, modulated the excitatory response to capsaicin.

Bradykinin

The excitation of nociceptive afferents innervating the rat knee joint evoked by bradykinin (9 nmol) in the presence of octreotide (10 nmol) was examined in 6 fibres from 3 normal knee joints (Figure 4.3B). The mean response evoked by bradykinin before octreotide was 5.6 ± 1.9 impulses s^{-1} and the total number of action potentials evoked was 128 ± 38 impulses. Five minutes after close arterial injection of octreotide the excitation evoked by bradykinin (9 nmol) was not significantly different, with a mean discharge frequency of 6.4 ± 3.0 impulses s^{-1} ($P = 0.51$; paired t-test) and a mean action potential discharge of 128 ± 70 ($P = 0.99$; paired t-test).

α, β - methylene ATP

The rapid excitation evoked by $\alpha\beta meATP$ (60 nmol) was examined before and after octreotide (10 nmol) in 6 fibres from 3 normal knee joints (Figure 4.3C). Before octreotide there was a mean increase in the discharge of 22.5 ± 14.1 impulses s^{-1} and the mean number of action potentials generated was 28 ± 14 impulses. When the same dose of $\alpha\beta meATP$ was repeated 5 minutes after close-arterial injection of octreotide (10 nmol) the response was not significantly different; the discharge frequency was 14.5 ± 9.2 impulses s^{-1} ($P = 0.29$; paired t-test) and a mean absolute discharge of 23 ± 18 impulses ($P = 0.59$; paired t-test).

Although octreotide and somatostatin-14 appeared not to influence either spontaneous firing or evoked responses of peripheral nociceptors, both were

biologically active, as demonstrated by their ability to reduce blood pressure. A typical vasodepressor response to octreotide (10 nmol) and somatostatin-14 (6 nmol) is shown in Figure 4.4.

4.4 DISCUSSION

This section of the thesis examined the ability of SOM and its analog octreotide to modulate nociceptive afferents innervating the normal and arthritic rat knee joint. Results show that neither SOM nor octreotide inhibited spontaneous discharge arising from normal or arthritic joints. Similarly, evoked responses to known algogens capsaicin, bradykinin, and $\alpha\beta$ meATP were not altered after close-arterial injection of SOM and octreotide.

Receptors for the neuropeptide SOM have been found in the rat DRG and therefore may play a role in the modulation of peripheral nociceptor signalling (see Introduction). In the vast majority of tissues the effect of SOM receptor activation is inhibitory, however several reports of excitatory effects of the neuropeptide have been noted (Randic & Miletic, 1978; Wiesenfeld-Hallin, 1985). Wiesenfeld-Hallin et al. (1985) in particular, attributed spinal cord excitability to SOM released from primary afferent C-fibres acting either locally on adjacent fibres or systemically once released into the circulation. In our model similar doses of SOM-14 or octreotide were unable to directly activate sensory afferents innervating either the normal or arthritic rat knee joint.

It has been reported that SOM, either exogenously applied or released from sensory terminals by anti-dromic stimulation, is able to inhibit the activation of mechanonociceptors innervating the rat knee joint (Heppelmann & Pawlak, 1997a;

Heppelmann & Pawlak, 1999). In our model we examined the effect of close arterial injection of SOM-14 and octreotide on spontaneously active afferents innervating the rat knee joint. There was no effect on the spontaneous discharge from afferents in either normal or arthritic knee joints. Additionally we found no effect on responses evoked by capsaicin, bradykinin or $\alpha\beta$ meATP after the close arterial injection of either SOM-14 or octreotide. Although there was no effect on spontaneous or evoked responses from nociceptors innervating the knee joint both SOM and octreotide were capable of producing a biological effect, detected as a vasodepressor response in the arterial blood pressure.

Although SOM-14 and octreotide do not appear to modulate spontaneous or chemically evoked discharge from primary afferents, recent reports have shown that SOM may still play a role in peripheral nociception. It has been shown that the SSTR_{2A} receptor is expressed on the peripheral terminals of primary afferents innervating rat skin (Carlton *et al.*, 2001a). Furthermore, *in vivo* following intraplantar injection of formalin, octreotide was able to diminish nociceptive behaviour such as licking and chewing of the injected limb. *In vitro*, octreotide was also able to inhibit bradykinin-mediated sensitisation to a noxious thermal stimulus (Carlton *et al.*, 2001a). In the present study the lack of effect of SOM-14 or octreotide could be due to the fact that the neurons expressing SSTR_{2A} receptors in the DRG are actually associated with medium to large diameter cells (Schulz *et al.*, 1998). Medium to large diameter cell bodies in the DRG are characteristic of myelinated fibres of the A δ and A β range. As the majority of afferents recorded in the present study were C-fibre polymodal nociceptors that arise from small diameter cells in the DRG it is possible that the SSTR receptor is not expressed on these fibres.

Even if SOM is not capable of modulating evoked C-fibre discharge, its release from the peripheral terminals of these same fibres plays an important role as both an anti-inflammatory and neuromodulator of peripheral nociceptors during acute inflammation. SOM released from peptidergic neurons is able to inhibit plasma extravasation at a remote site (Than *et al.*, 2000). Therefore it is likely that during neurogenic inflammation SOM is released together with pro-inflammatory neuropeptides such as substance P and CGRP. It is the relative levels of SOM and substance P/CGRP that control the release of pro-inflammatory cytokines (Weinstock & Elliott, 1998), such as interferon γ (IFN γ) and tumour necrosis factor (TNF α), that are capable of sensitising nociceptors and subsequently determine the nociceptive threshold of sensory afferents. Proof of this hypothesis has already been shown for mechanonociceptors innervating the normal knee joint (Heppelmann & Pawlak, 1999) and in peripheral cutaneous C-fibre polymodal nociceptors (Carlton *et al.*, 2001b) that become sensitised to activation by a noxious movement or thermal stimulus in the presence of the SOM antagonist cyclo-somatostatin.

The lack of inhibition of the spontaneous and evoked excitations of articular nociceptors may be due to the bolus injection used for SOM and the algogenic mediators. The administration SOM or octreotide as a bolus injection may not permit the peptide to be present at an adequate concentration over a period of time sufficient to alter the balance in tonically expressed pro- and anti-inflammatory neuropeptides. Similarly, all the models detailed above involved acute inflammation to sensitise peripheral afferents, so it may be that under conditions of chronic inflammation the equilibrium shifts so far towards pro-inflammatory peptides that a single bolus injection of SOM is unable to produce a significant inhibition. This

would fit with clinical trials in RA patients who require prolonged treatment with SOM-14 to produce analgesia (Coari *et al.*, 1995; Fioravanti *et al.*, 1993; Fioravanti *et al.*, 1995; Russo *et al.*, 1997). Further work could be undertaken to examine the effect of prolonged treatment with SOM or octreotide on the increased number of spontaneously active fibres or the increased response to bradykinin in C-fibre polymodal nociceptors innervating arthritic joints. This could be accomplished by the implantation of osmotic mini-pumps, administering SOM over a prolonged period.

Once the distribution of all SSTR receptors in the periphery is known, the targeting of these receptors with the aim of altering the balance of pro- and anti-inflammatory mediators during chronic inflammation may provide an effective treatment for conditions such as osteo- and rheumatoid arthritis.

TABLES AND FIGURES

Figure 4.2 – Effect of octreotide on spontaneous discharge in afferents innervating normal and arthritic rat knee joints

Basal spontaneous activity in peripheral nociceptors innervating A) normal (n=5) and B) arthritic (n=3) rat knee joints before and for 60 s following close arterial injection of octreotide (10 nmol). Data represents the mean \pm sem.

Octreotide did not affect spontaneous discharge in either normal or arthritic knee joints.

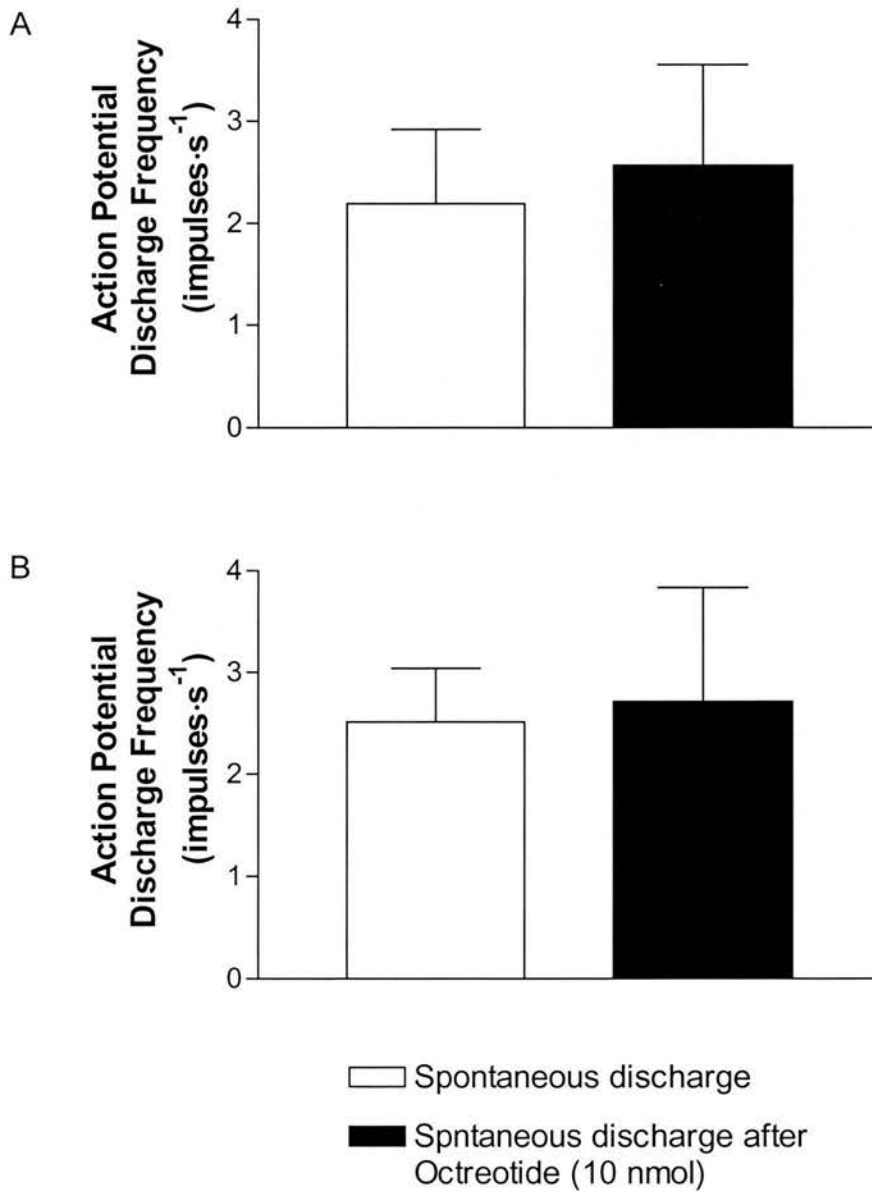


Figure 4.3 – Evoked responses to capsaicin, bradykinin, and $\alpha\beta$ meATP following octreotide in normal rat knee joints

Mean action potential discharge frequency and total number of action potentials evoked by A) capsaicin (3 nmol; 12 fibres from $n=5$ animals) B) bradykinin (9 nmol; 6 fibres from $n=3$ animals) and C) $\alpha\beta$ meATP (60 nmol; 6 fibres from $n=3$ animals) before and 5 minutes following close arterial injection of octreotide (10 nmol). Data represent mean \pm sem.

Octreotide does not alter the evoked afferent excitations by capsaicin, bradykinin, or $\alpha\beta$ meATP.

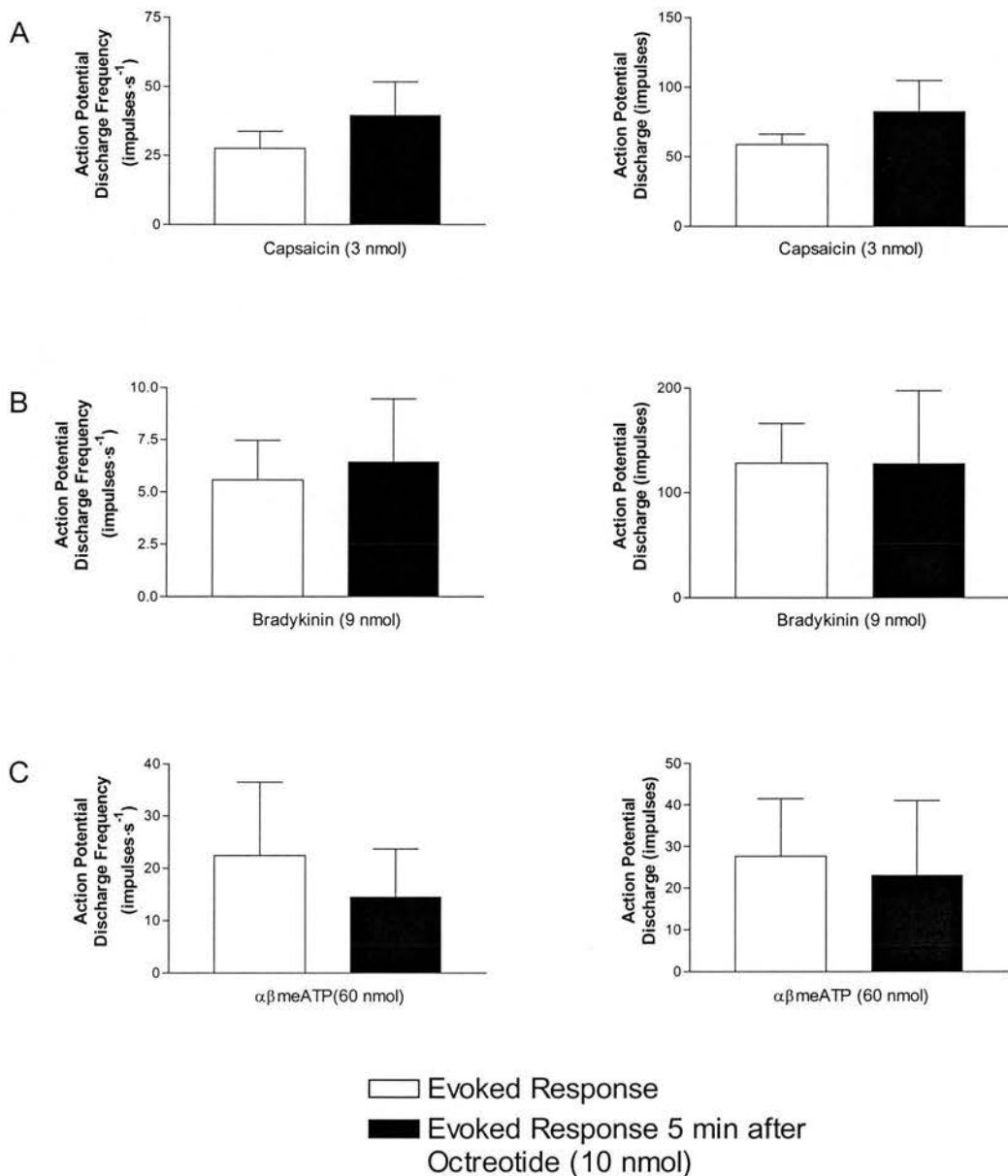
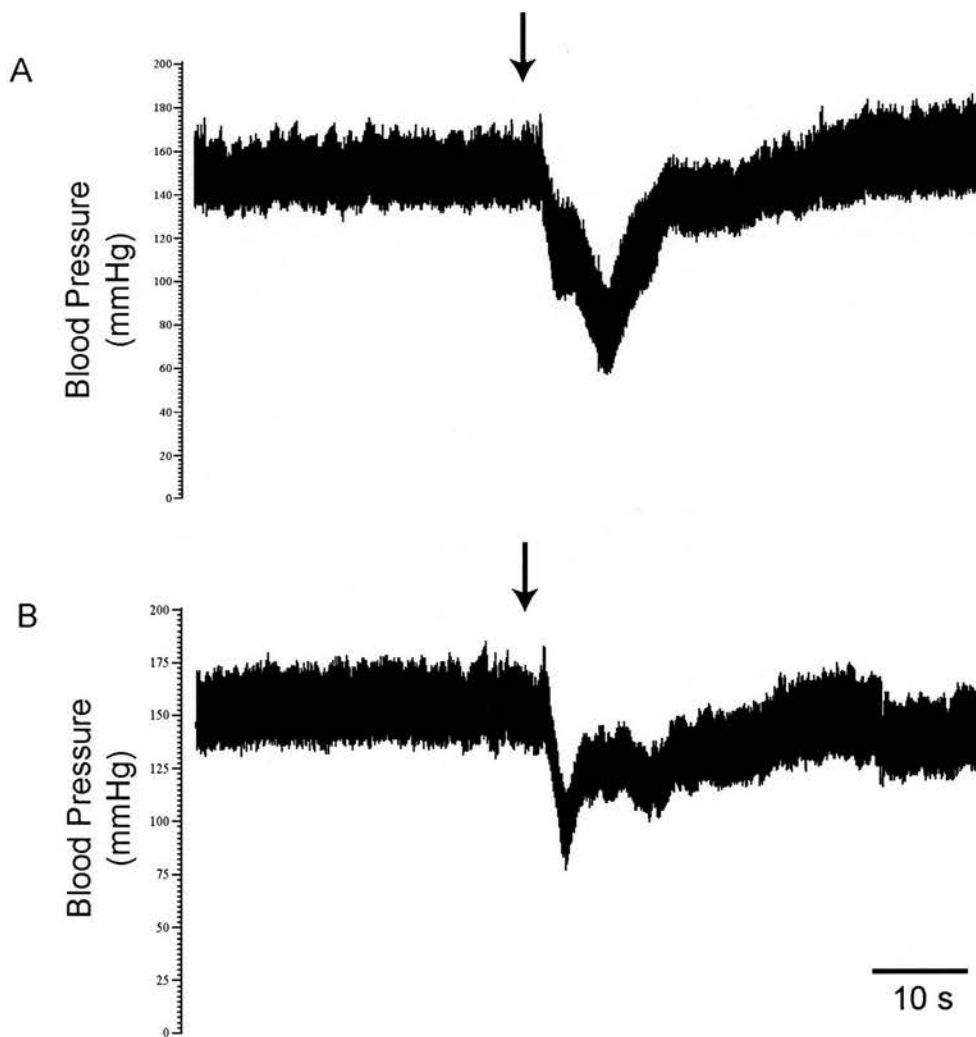


Figure 4.4 – Blood pressure response evoked by octreotide and somatostatin-14 in a normal rat.

The vasodepressor response evoked by the close arterial injection (at arrows) of A) octreotide (10 nmol) and B) somatostatin-14 (6 nmol) in a normal rat.

Both octreotide and somatostatin induce a fall in blood pressure within seconds of injection confirming that they were biologically active.



5. Effect of anandamide on knee joint nociceptors

5.1 INTRODUCTION

The cannabis sativa plant has been used therapeutically and recreationally for thousands of years, but very little research was done on the properties of the plant until the isolation of the main psychoactive constituent, Δ^9 (-) tetrahydrocannabinol (THC) by Gaoni and Mechoulam in 1964 (see detailed review by Mechoulam & Hanus, 2000). With the development of high affinity analogs of THC, a specific cannabinoid receptor (CB₁) in the CNS was characterised (Devane *et al.*, 1988) and cloned (Matsuda *et al.*, 1990). This was followed by the characterisation of a second receptor (CB₂) present in the periphery (Munro *et al.*, 1993). Recent reports have shown that the CB₁ receptor is not restricted to the CNS and is also expressed in the periphery (Ahluwalia *et al.*, 2000) and furthermore, CB₁ receptors are produced in the cell bodies of sensory neurons in the dorsal root ganglia and are transferred by axonal transport to the peripheral terminals of primary nociceptors (Hohmann & Herkenham, 1999). The majority of the properties attributed to cannabis, in particular its antinociceptive and antihyperalgesic effects are mediated through its action at the CB₁ receptor either centrally (Richardson *et al.*, 1998a) or in the periphery (Calignano *et al.*, 1998; Richardson *et al.*, 1998b).

With the discovery of a receptor for cannabinoid compounds it was assumed that there must be an endogenous compound capable of activating the receptor and producing similar effects to THC (Martin *et al.*, 1999; Mechoulam *et al.*, 1998). The first endogenous cannabinoid or *endocannabinoid*, arachidonoyl ethanolamide, was isolated from pig brain and was given the name anandamide - from the Sanskrit word “ananda” meaning “bliss” (Devane *et al.*, 1992). Anandamide binds with moderate

affinity to the CB₁ receptor and low affinity at the CB₂ receptor (Khanolkar & Makriyannis, 1999) and it possesses many of the properties of THC including antinociceptive (reviewed in Pertwee, 2001) and antihyperalgesic action (Richardson *et al.*, 1998a).

ANANDAMIDE AS A NEUROMODULATOR IN NOCICEPTION

An important putative role for anandamide is as a neuromodulator of nociception. Anandamide behaves as a classic neurotransmitter in that it is synthesised from membrane phospholipids and released on demand by neurons after being triggered by Ca²⁺ influx or cell injury. Mechanisms exist for rapid enzymatic degradation by fatty acid amide hydrolase (FAAH) and for re-uptake by a specific anandamide membrane transporter (Di Marzo, 1999). Once bound to the G-protein-coupled CB₁ receptor, anandamide is capable of modulating neuronal membrane permeability to Ca²⁺ and K⁺ ions and inhibiting the activity of adenylate cyclase (reviewed in Di Marzo *et al.*, 1998b and Howlett & Mukhopadhyay, 2000).

A role of anandamide in nociception was suggested when it was shown that nociceptive responses were increased in the presence of the specific CB₁ antagonist, SR141716A (Chapman, 1999). However, this evidence is not in accord with recent studies in CB₁ knockout mice, which showed hypoalgesia in the hot-plate test, and in addition it was still possible to induce analgesia with cannabinoids (Zimmer *et al.*, 1999). Although it is known that endocannabinoids are capable of inducing intra-cellular signalling via both receptor and non-receptor pathways (Felder *et al.*, 1993; Felder *et al.*, 1992) it was proposed that anandamide might produce effects through activation of an entirely different receptor population, namely vanilloid receptors.

Recent reports have linked anandamide with the recently cloned vanilloid receptor subtype 1 (VR₁) which is sensitive to capsaicin (Caterina *et al.*, 1997) and is antagonised by capsazepine (Bevan *et al.*, 1992; Szallasi & Blumberg, 1999). Di Marzo *et al.* (1998) used the structural similarity between synthetic agonists of vanilloid receptors and anandamide to predict interactions between the cannabinoid and vanilloid systems. They showed that the capsaicin analog olvanil, which exhibits some analgesic properties, is able to bind to and inhibit the anandamide membrane transporter thereby preventing the re-uptake of anandamide (Di Marzo *et al.*, 1998a). Conversely, it has also been shown that anandamide is a vasorelaxant (Randall & Kendall, 1998a; Randall & Kendall, 1998b) and this vasodilatation results from VR₁-mediated release of calcitonin-gene-related peptide (CGRP) from perivascular sensory nerves (Zygmunt *et al.*, 1999).

The present investigation used electrophysiological methods to examine the affect of anandamide on the function of peripheral nociceptors innervating normal and chronically inflamed arthritic knee joints. Both CB₁ and VR₁ receptors are co-localised in small diameter cell bodies in the DRG and therefore are likely present on the peripheral terminals of joint afferents, a study was undertaken to test whether anandamide affects spontaneous or chemically evoked discharge in normal knee joints, or in the joints of rats with FCA-induced experimental arthritis, via activation of peripheral CB₁ receptors. In addition the ability of anandamide to directly activate peripheral nociceptors through activation of the VR₁ receptor was also examined. The hypothesis was that since the VR₁ receptor is readily desensitised by stimulation the analgesic effect of anandamide may be a result of its action on peripheral nociceptors to diminish their responsiveness.

5.2 METHODS

AFFERENT NERVE RECORDING *IN VIVO*

Arthritis induction and neural recordings were carried out as described in Chapter 2.2. Experiments were conducted in a total of 16 normal and 10 arthritic Wistar rats (body weight range 230-470 g; mean \pm sem. 375 ± 12 g). Arthritis was induced 14-35 days before the animals were used in electrophysiological studies. Animals displayed mild but persistent unilateral arthritis as characterised by a significant increase in the diameter of the injected joint (injected 10.5 ± 0.1 mm; uninjected 9.8 ± 0.1 mm; $n=10$; $P=0.0001$, paired t-test). Animals were anaesthetised with pentobarbitone (60 mg kg^{-1} i.p; deep anaesthesia maintained by infusing $0.5 \text{ mg kg}^{-1} \text{ min}^{-1}$ i.v.) then prepared surgically as detailed in Chapter 2.2. Extracellular neural activity in a filament of the MAN was recorded using bipolar platinum-iridium electrodes. Collected data was analysed off-line using Spike2 software. Drugs were administered by close arterial injection into the right femoral cannula in a total volume of 0.1 ml.

5.3 RESULTS

CHARACTERISATION OF AFFERENT NERVE FIBRES

Afferent neural discharge was recorded from a total of 48 individual fibres from 16 normal knee joints and 24 individual fibres innervating 10 arthritic knee joints. The afferents were classified as either C-fibre polymodal nociceptors or A δ mechanonociceptors based on their conduction velocities, spike duration, mechanosensitivity and responsiveness to capsaicin as described in Chapter 2.2. In

normal joints, 41/48 (85%) of fibres were classified as C-fibre polymodal nociceptors and 7/48 (15%) were classified as A δ mechanonociceptors. In arthritic joints, 21/24 (87%) of afferents were classified as C-fibres and 3/24 (13%) were classified as A δ fibres. There was no significant difference in the proportion of C and A δ between normal and arthritic joints ($P = 1.00$ and $P = 1.00$ respectively, Fisher's exact test). On-going (spontaneous) discharge occurred in 15/48 fibres (31%) in normal joints, with a mean discharge frequency of 0.01 ± 0.01 impulses s^{-1} and in arthritic joints 11/24 (46%) afferents showed spontaneous activity with a mean discharge frequency of 0.04 ± 0.01 impulses s^{-1} . There was no significant difference in the mean discharge frequency recorded from normal and arthritic knee joints ($P = 0.12$; unpaired t-test).

ANANDAMIDE-EVOKED ACTIVATION OF KNEE JOINT AFFERENTS

Proportion of afferents activated by anandamide

A single close arterial injection of anandamide (8.6 nmol – 2900 nmol) evoked an increase in afferent discharge that was rapid in onset and of a short duration (Figure 5.1). The excitatory response to anandamide was present in 21/41 (51%) C-fibre polymodal nociceptors but was not seen in any of the A δ -mechanonociceptors studied (0/7; 0%) in normal knee joints. Similarly, in arthritic joints anandamide evoked a response in 10/21 (48%) C-fibre polymodal nociceptors but did not excite A δ mechanonociceptors (0/3; 0%), there was no significant difference in the proportion of afferents innervating normal and arthritic joints (C-fibres: $P = 1.00$, A-fibre: N/A; Fisher's exact test; Figure 5.2). Anandamide was injected in a suspension of soya oil and water (1:4) and injection of vehicle (0.1 ml)

had no effect in these fibres with a mean discharge frequency of -0.1 ± 0.1 impulses \cdot s $^{-1}$ (n=5).

Dose-related increase in the nociceptor action potential discharge evoked by anandamide

The excitation evoked by anandamide in the C-fibre polymodal nociceptors innervating the knee joint was dose-dependent for both the total action potential discharge (Figure 5.3A) and the discharge frequency (Figure 5.3B). Due to the limited solubility of anandamide, the maximum dose that could be administered was 2900 nmol - the stock solution supplied. For this reason the maximum response was that evoked by 2900 nmol. As a result it was impossible to calculate exact ED₅₀ values and so *apparent* ED₅₀ values were calculated, based on the response to 2900 nmol being “maximum”. The mean apparent ED₅₀ for the activation of C-fibres by anandamide in normal joints (n=3) was 1.1 ± 0.7 μ mol and 3.0 ± 0.9 μ mol in arthritic joints (n=3) – this difference was not statistically significant (P = 0.20, Mann-Whitney)

Features of the anandamide-evoked response

The onset delay, duration, and magnitude of the response evoked by a sub-maximal dose (860 nmol) of anandamide were determined in both normal (n=15 individual fibres from 5 animals) and arthritic knee joints (n=11 individual fibres from 6 animals). The magnitude of the response was calculated both in terms of the number of action potentials generated in an evoked discharge, and the discharge frequency. Figure 5.4 shows a typical excitatory response to anandamide (860 nmol) recorded from nociceptive afferents innervating normal and arthritic knee joints.

Response latency

Anandamide rapidly excited afferents innervating the knee joint (Figure 5.5A). In normal joints the mean response latency to 860 nmol of anandamide was 4.5 ± 0.5 s and the corresponding figure for arthritic joints was 5.2 ± 0.4 s. There was no significant difference in the response latency between normal and arthritic joints ($P = 0.28$; unpaired t-test)

Response duration

The response to anandamide was short lasting in both normal ($n=5$) and arthritic ($n=6$) joints with mean response durations of 4.8 ± 1.0 s and 3.9 ± 1.1 s respectively. Figure 5.5B illustrates the response duration; there was no significant difference between normal and arthritic animals ($P = 0.54$; unpaired t-test)

Magnitude of the response

The magnitude of the excitatory response elicited by anandamide was examined in terms of the discharge frequency (Figure 5.5C) and total number of action potentials generated (Figure 5.5D). In normal joints ($n=5$) the mean discharge frequency was 7.2 ± 2.3 impulses·s⁻¹ where the mean number of action potentials generated was 67 ± 25 . Arthritic joints ($n=6$) had a mean discharge frequency of 9.7 ± 3.0 impulses·s⁻¹ and a mean action potential discharge of 50 ± 24 impulses. Once again there was no significant difference between normal and arthritic joints for the frequency of the response or the number of action potentials produced ($P = 0.53$ and $P = 0.65$ respectively; unpaired t-test).

EFFECT OF ANANDAMIDE ON EXCITATORY RESPONSES EVOKED BY ATP, BRADYKININ AND CAPSAICIN

As part of the routine pharmacological characterisation of the joint afferents, injections of algogens in doses capable of activating polymodal nociceptors were administered during each experiment. These algogens included capsaicin, bradykinin, and ATP or its stable analog, $\alpha\beta\text{meATP}$. They were given before and after multiple, increasing doses of anandamide (8.6 nmol – 2900 nmol), thereby permitting the examination of the effect of anandamide on the evoked responses – results are shown in Table 5.1.

In normal joints the response to bradykinin (9 nmol) was 1.7 ± 0.7 impulses·s⁻¹, and after anandamide it was not significantly different with a response of 1.3 ± 0.6 impulses·s⁻¹ (n=8 individual fibres from 2 animals; P = 0.14, Wilcoxon). The response to ATP (2000 nmol) was unchanged after the administration of anandamide (P = 0.69, Mann-Whitney) with a response of 14.8 ± 6.0 impulses·s⁻¹ prior to anandamide and 11.2 ± 4.7 impulses·s⁻¹ following (n=5 individual fibres from 2 animals). In arthritic joints after anandamide, there was no significant difference in the response to bradykinin or ATP (P = 0.43 and P = 0.31 respectively; Wilcoxon), with evoked discharge averaging 2.2 ± 0.6 impulses·s⁻¹ and 1.3 ± 0.5 impulses·s⁻¹ (n=6 individual fibres from 3 animals) before and after bradykinin respectively, and 2.3 ± 1.5 impulses·s⁻¹ and 6.5 ± 2.5 impulses·s⁻¹ (n=4 individual fibres from 3 animals) before and after ATP respectively.

The response to capsaicin (9 nmol) was also examined before and after anandamide. In normal joints, the evoked discharge averaged 21.0 ± 7.6 impulses·s⁻¹

before, and 12.1 ± 3.5 impulses·s⁻¹ after anandamide, and this difference was not statistically significant ($P = 0.46$, Wilcoxon, $n=12$ from 4 animals). Similarly, no difference was seen in the arthritic joints, with responses of 11.6 ± 2.0 impulses·s⁻¹ before and 8.1 ± 2.2 impulses·s⁻¹ after anandamide ($n=11$ from 6 animals; $P = 0.28$, Wilcoxon).

COMPARISON OF THE CAPSAICIN AND ANANDAMIDE-EVOKED RESPONSES IN NORMAL KNEE JOINTS

It was proposed that, in addition to the activation of CB₁ receptors, some of the actions of anandamide might be attributable to the activation of vanilloid VR₁ receptors (see Introduction). The afferent nociceptive excitation generated by a sub-maximal dose of anandamide (860 nmol) was compared to that produced by a standard dose of capsaicin (9 nmol). Figure 5.1 shows the typical responses evoked by anandamide and capsaicin, and it can be seen that, generally speaking, the two responses are similar. Furthermore all the afferents activated by anandamide (21 afferents from 16 animals) were also excited by capsaicin.

The examination of the components of the responses, in terms of the response latency, duration, and magnitude, for capsaicin and anandamide are shown in Figure 5.6. The delay in onset of the response was longer following anandamide in comparison with capsaicin (4.5 ± 0.5 s and 2.6 ± 0.3 s respectively), and this difference was statically significant ($P = 0.002$; unpaired t-test; Figure 5.6A). Both drugs produced excitations with short durations of 8.2 ± 4.5 s for capsaicin and 4.8 ± 1.0 s for anandamide and the difference was not significant ($P = 0.37$; unpaired t-test; Figure 5.6B). The magnitude of the response is obviously dependent on the dose

administered, and the doses were chosen to generate similar levels of afferent excitation. There was no significant difference in the magnitude of the excitatory responses when the total number of action potentials generated by either capsaicin or anandamide ($P = 0.11$; unpaired t-test; Figure 5.6D) was measured. However, the mean discharge frequency evoked by capsaicin was significantly larger than that seen following anandamide ($P = 0.02$; unpaired t-test; Figure 5.6C).

RESPONSE TO ANANDAMIDE IS ABOLISHED BY THE VR₁ ANTAGONIST, CAPSAZEPINE

In order to confirm the activation of polymodal nociceptors by anandamide was attributable to activation of VR₁ receptors, the effects of both capsaicin (9nmol) and anandamide (2900 nmol) were examined in the presence of the VR₁ antagonist capsazepine (1 mg kg⁻¹). In a normal rat knee joint, capsazepine completely abolished the afferent response to both capsaicin and anandamide (Figure 5.7). Capsazepine has a short half life in vivo and therefore the effect was short-lasting - the excitatory response to both drugs recovered after 20 minutes. Furthermore the effect was specific to capsaicin and anandamide, as responses to both bradykinin (9 nmol) and $\alpha\beta$ meATP (60 nmol) were not affected by capsazepine (Table 5.2).

5.4 DISCUSSION

The primary, and somewhat unexpected finding from this *in vivo* study is that close intra-articular injection of anandamide causes a dose-dependent rapid onset, short-lasting excitation in a sub-population of polymodal nociceptors in normal and arthritic rat knee joints.

Anandamide is an agonist at CB₁ and CB₂ receptors, and both types are present in the periphery - CB₁ receptors are localised to sensory nerves and the CB₂ receptors are expressed on immune cells, such as mast cells, B-cells and natural killer cells. The ability of anandamide to activate nociceptors was unexpected and appears to be inconsistent with previously reported anti-nociceptive actions associated with CB₁ receptor activation. Cannabinoids act on the CB₁ receptor both centrally in the brain and spinal cord, and peripherally in the dorsal root ganglion, to cause analgesia (reviewed in Fuentes *et al.*, 1999; Pertwee, 2001; Walker *et al.*, 1999). In the present study the focus was on determining what effect anandamide has on peripheral nociceptive nerve terminals located in an articular joint. Analgesic effects of the CB₁ receptor appear to result from relatively low doses of cannabinoids, and the high doses of anandamide required to activate peripheral nociceptors in our study are likely to result from actions on a different pharmacological receptor.

The rapid onset of the response to anandamide eliminates a number of potential indirect mechanisms, such as activation of the CB₂ receptor on immune cells triggering the release of algogenic mediators. Furthermore, even allowing for the rapid metabolism of anandamide to arachadonic acid and subsequently to eicosanoids, the latency of onset of the response is too short to result from the action of a metabolite. The rapid response is not characteristic of G-protein coupled

receptor activation; rather it is more representative of the response of a ligand-gated ion-channel, such as the VR₁ receptor channel (Szallasi & Blumberg, 1999).

It is now known that anandamide is a full agonist at the human VR₁ receptor when expressed in HEK293 cells (Smart *et al.*, 2000), and the vasodilatory action of anandamide is a result of VR₁ mediated release of CGRP (Zygmunt *et al.*, 1999). The afferents activated by anandamide were all sensitive to capsaicin, but the corollary did not apply as only 64% and 72% of capsaicin sensitive afferents were activated by anandamide in normal and arthritic joints respectively. The VR₁ receptor is expressed on approximately 88% of small to medium neurones in the dorsal root ganglion (Michael & Priestley, 1999). These are the cell bodies for thinly myelinated (A δ) or unmyelinated (C) fibres running from the periphery. Although the VR₁ receptor is not present on all sensory afferents, our study did not reveal afferents that are sensitive to anandamide but insensitive to capsaicin. Furthermore, the present work showed that activation of capsaicin sensitive sensory nerves *in vivo* by anandamide can be abolished by the VR₁ antagonist capsazepine (1 mg kg⁻¹). Therefore, excitation of peripheral nociceptors by high doses of anandamide appears to involve a VR₁-dependent mechanism. In addition, it can be concluded that if cannabinoid receptors are present on peripheral terminals they are not excitatory.

Capsaicin, even in small doses, desensitised the VR₁ receptor in our preparation, whereas anandamide did not cause desensitisation either to capsaicin or itself in the doses tested. Future experiments should examine the afferent response to anandamide after a desensitising dose of capsaicin (>86 nmol).

Low doses of cannabinoids can produce anti-hyperalgesia and anti-nociception when acting both centrally and peripherally (Johanek *et al.*, 2001). In

the present study higher doses of anandamide were examined to determine the effect on responses evoked by other algogens commonly used to characterise primary nociceptive afferents. The results show that responses to three of these (capsaicin, bradykinin, and ATP) were not significantly different after anandamide (860, 2900 nmol). Therefore anandamide did not reduce the chemosensitivity of primary sensory afferents. More detailed experiments are required to determine whether the high threshold mechanosensitivity or thermo-sensitivity of these afferents is affected by high doses of anandamide.

Recently there has been debate in the literature that the concentrations of anandamide required to activate VR₁ are not physiologically relevant (Smart & Jerman, 2000; Szolcsanyi, 2000a; Szolcsanyi, 2000b; Zygmunt *et al.*, 2000). It is still not clear at what concentration anandamide might be present at peripheral nerve terminals. To determine this would require direct measurement of anandamide concentration at peripheral terminals requiring techniques that are not currently available. It is possible that levels of locally produced ligand are sufficient for activation of nearby sensory nerve terminals. Similarly, it is possible that an unknown endogenous compound, similar to anandamide in structure with a higher affinity for the VR₁ receptor, exists. Regardless of whether the ability of anandamide to activate the vanilloid receptor has any physiological significance, it does have pharmacological relevance with regard both to the pharmacological profile of anandamide and to the development of novel vanilloid receptor ligands.

TABLES AND FIGURES

Table 5.1 – Effect of anandamide on evoked excitation from nociceptive afferents by ATP, bradykinin and capsaicin.

Comparison of the ability of a close-arterial injection of anandamide (860 nmol) to inhibit evoked excitation from articular nociceptive afferents innervating the normal and arthritic rat knee joint. Results are the mean afferent discharge frequency \pm sem (impulses s⁻¹).

Anandamide at high doses is not able to inhibit the excitation of nociceptive afferents by ATP, bradykinin, or capsaicin in normal and arthritic joints.

	<u>Normal Knee Joints</u>		<u>Arthritic Knee Joints</u>	
	Discharge frequency evoked before anandamide (860 nmol)	Discharge frequency evoked after anandamide (860 nmol)	Discharge frequency evoked before anandamide (860 nmol)	Discharge frequency evoked after anandamide (860 nmol)
ATP (2000 nmol)	14.8 \pm 6.0 (n=5)	11.2 \pm 4.7 (n=5)	2.3 \pm 1.5 (n=4)	6.5 \pm 2.5 (n=4)
Bradykinin (9 nmol)	1.7 \pm 0.7 (n=8)	1.3 \pm 0.6 (n=8)	2.2 \pm 0.6 (n=5)	1.3 \pm 0.5 (n=5)
Capsaicin (9 nmol)	21.0 \pm 7.6 (n=8)	12.1 \pm 3.5 (n=8)	11.6 \pm 2.0 (n=11)	8.1 \pm 2.2 (n=11)

Table 5.2 – Responses evoked by bradykinin and $\alpha\beta$ meATP before and after capsazepine

Comparison of the excitation evoked from articular nociceptive afferents innervating the normal and arthritic rat knee joint by bradykinin and $\alpha\beta$ meATP following the close-arterial injection of capsazepine (1 mg kg^{-1}).

Capsazepine does not inhibit excitation evoked from nociceptive afferents by bradykinin or $\alpha\beta$ meATP.

		Response	After Capsazepine (1 mg kg^{-1}; i.a.)
Bradykinin (9 nmol; n=1)	Discharge Frequency (impulses s^{-1})	1.72	1.05
	Total Discharge (impulses)	48	50
$\alpha\beta$meATP (60 nmol; n=1)	Discharge Frequency (impulses s^{-1})	10.70	10.65
	Total Discharge (impulses)	27	21

Figure 5.1 – Anandamide excites a population of sensory afferents in the normal rat knee joint.

Multi-fibre neural discharge (left panel) recorded from a portion of the MAN showing the response evoked from individual fibres (right panel) by close-arterial injection of A) anandamide (860 nmol, which activated fibres 1, 3, and 4), B) capsaicin (9 nmol, activated fibres 1, 3-6), C) bradykinin (9 nmol, activated fibres 3 and 4), and D) $\alpha\beta$ meATP (60 nmol, activated fibres 2 and 6). Anandamide response shown in comparison to data presented earlier (see Figure 3.3). Filters were set to allow only discharge above noise level to be captured. Individual fibres are shown on the right panel.

Anandamide activates a population of capsaicin sensitive afferents.

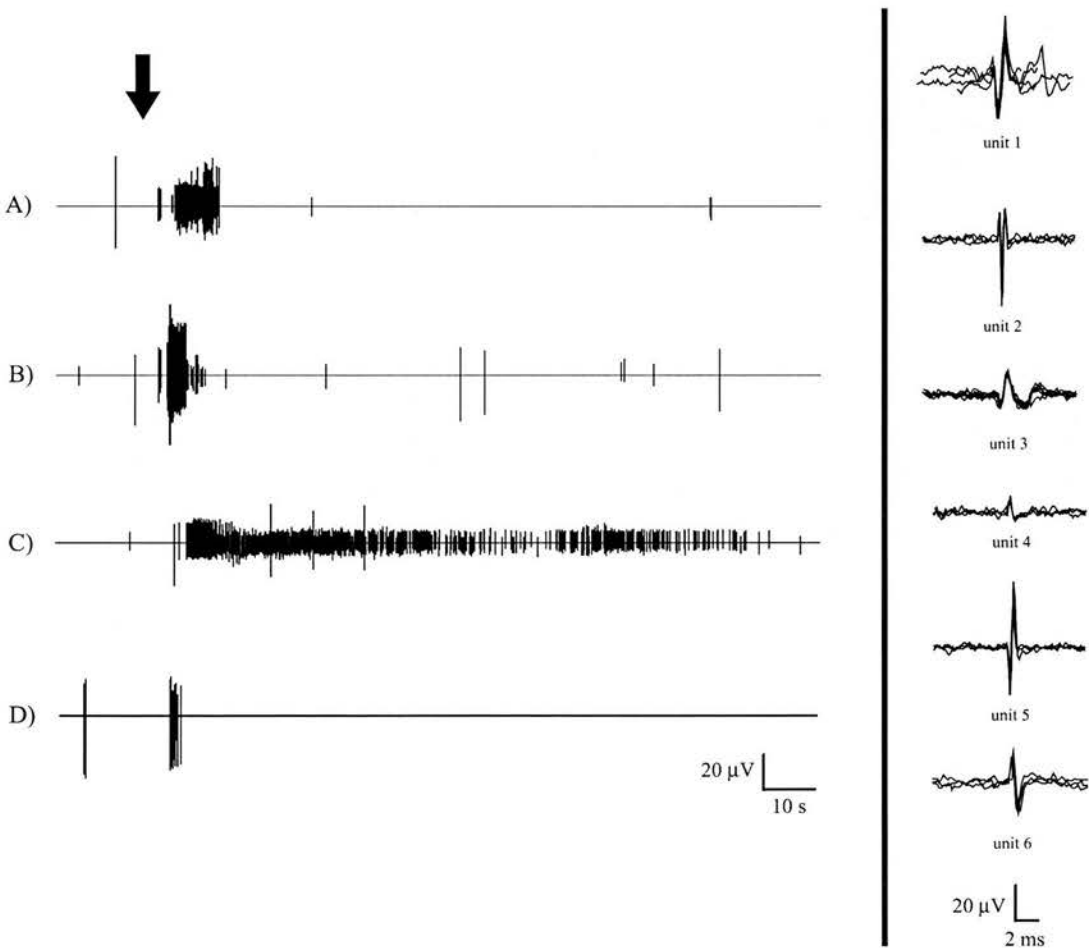


Figure 5.2 – Proportion of C and A δ fibres activated by anandamide

Proportion of afferent fibres both A) C-fibre polymodal nociceptors and B) A δ mechanonociceptors activated by anandamide (860 nmol-2900 nmol; close i.a.) in normal (n=41 individual fibres from 16 animals) and arthritic (n=21 individual fibres from 10 animals) rat knee joints.

Anandamide activated a population of C-fibre polymodal nociceptors in both normal and arthritic knee joints but did not activate any of the A δ mechanonociceptors sampled.

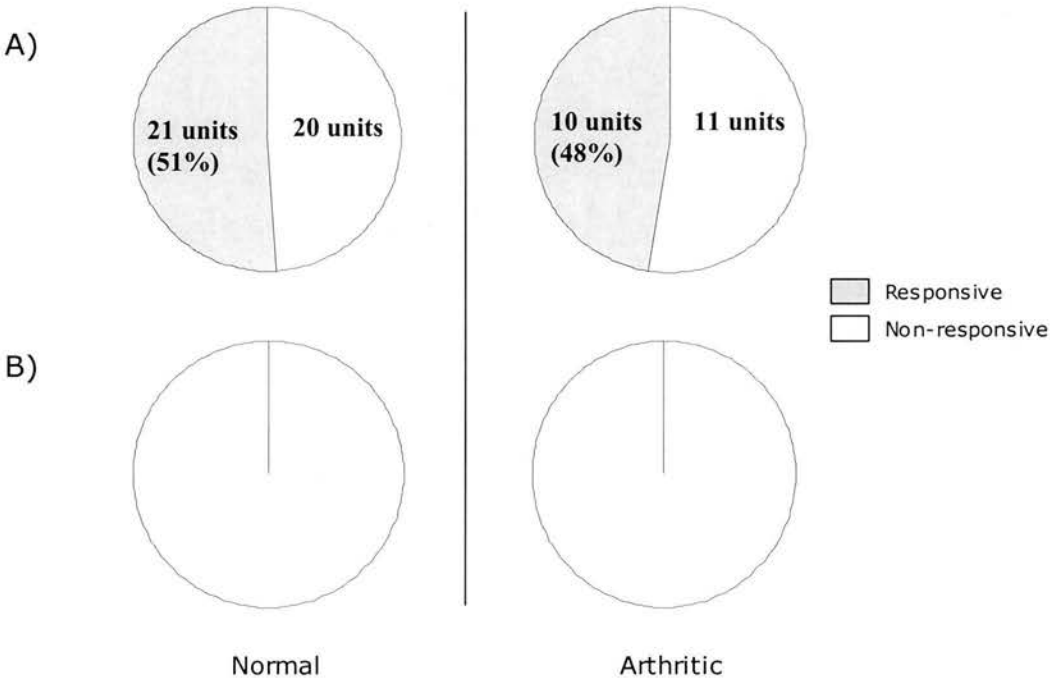


Figure 5.3 – Anandamide activates sensory afferents in a dose-dependent manner

Typical log dose response curves displaying the dose-related increase in A) the total number of action potentials produced (impulses) and B) discharge frequency (impulses s^{-1}) evoked by close arterial injection of anandamide (8.6 – 2900 nmol) in a normal knee joint.

Anandamide evoked action potential discharge in a dose-dependent manner from nociceptive afferents of the MAN

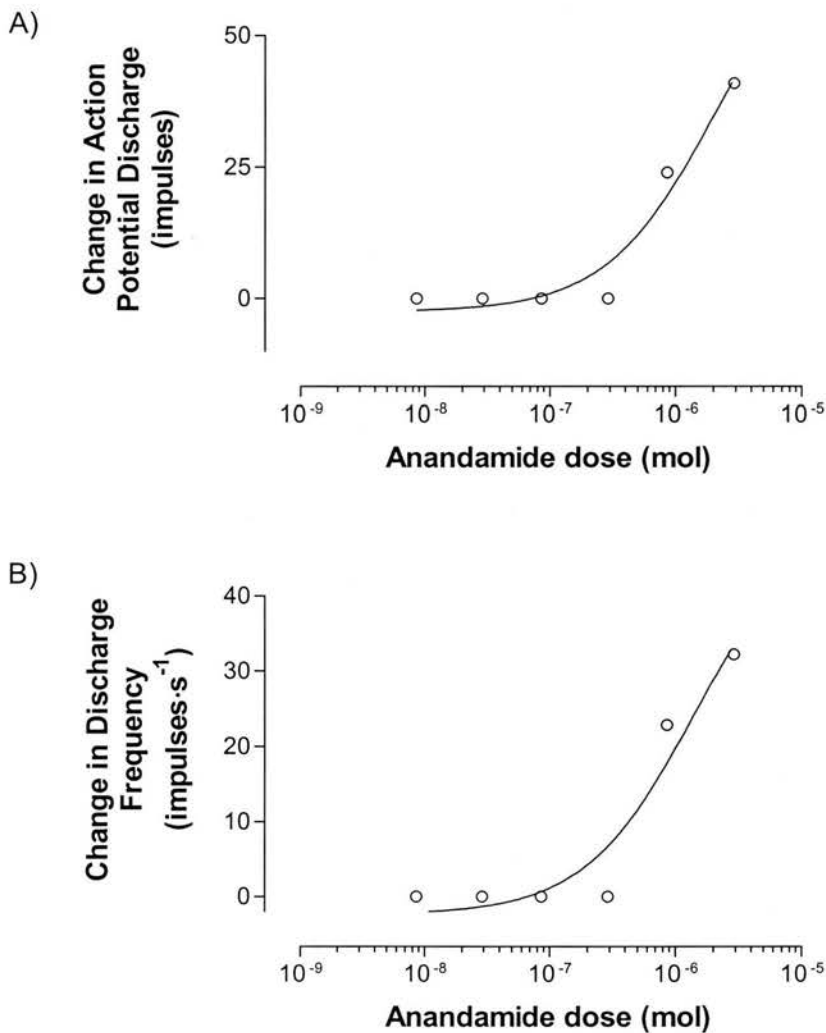


Figure 5.4 – Excitatory effects of anandamide on afferent discharge recorded from normal and arthritic knee joints.

Neural discharge showing the response evoked from individual nociceptive (capsaicin positive) fibres (right panel) recorded from the MAN innervating the A) normal and B) arthritic rat knee joint by close-arterial injection of anandamide (860 nmol; arrow). The upper panels show the arterial blood pressure and the lower panel is a bar graph representing the pooled discharge of the individual fibres. Individual fibres are shown in the right panel. Discharge is displayed in 1 s bins.

Close arterial injection of anandamide induces a rapid, short duration excitation from articular afferents innervating both normal and arthritic joints.

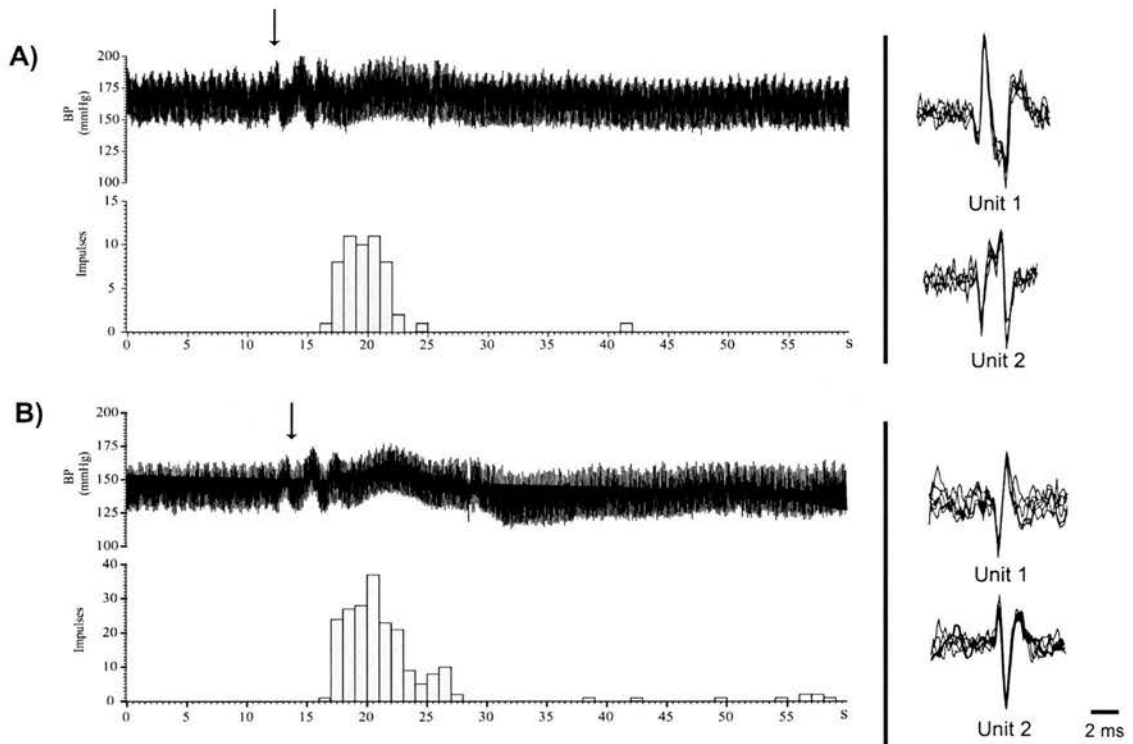


Figure 5.5 – Comparison of the excitatory response recorded from nociceptive afferents in normal and arthritic rat knee joints following injection of anandamide

Comparison of the A) latency to onset, B) response duration, C) change in action potential discharge frequency and D) change in total action potential discharge following a close arterial injection of anandamide (860 nmol). Data shown represents the mean \pm sem for responses from normal (n=15 individual fibres from 5 animals) and chronically arthritic (n=11 individual fibres from 6 animals) joints.

There is no significant difference in any facet of the response in comparisons between normal and arthritic joints.

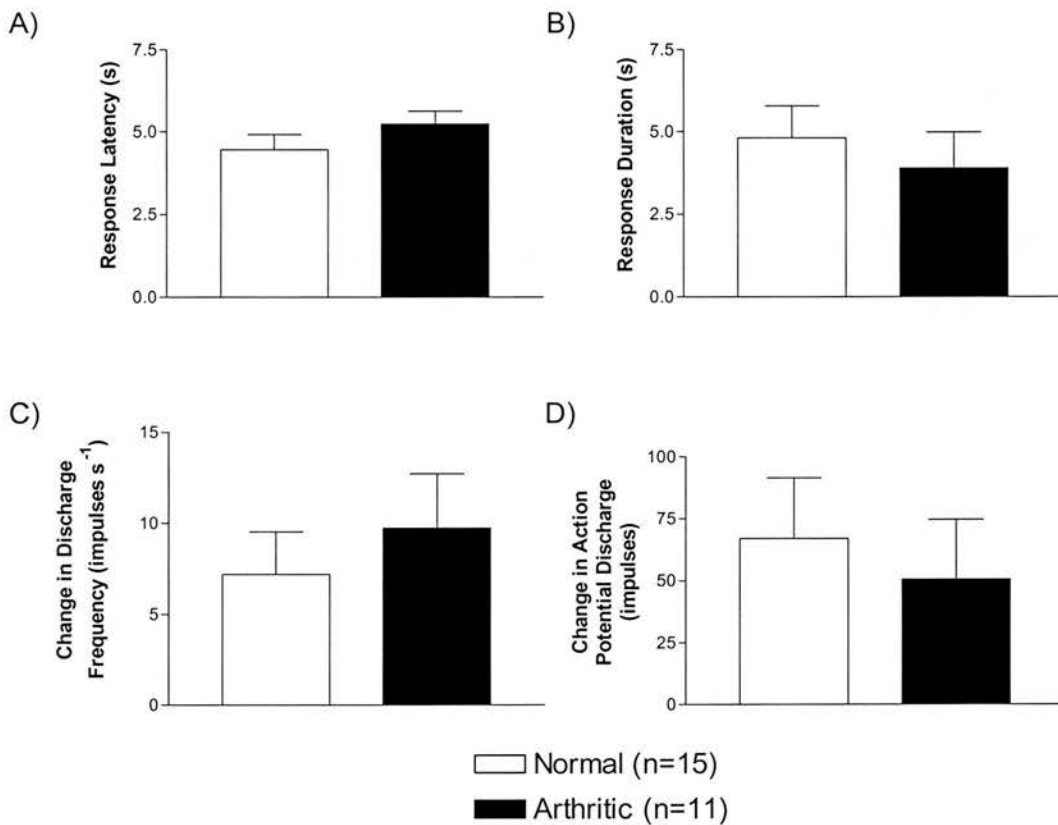


Figure 5.6 – Comparison of the excitation evoked by capsaicin and anandamide from articular nociceptive afferents innervating normal rat knee joints.

Comparison of the A) latency to onset, B) duration of the response, C) action potential discharge frequency and D) total number of action potentials elicited following a close arterial injection of sub-maximal doses of either capsaicin (9 nmol) or anandamide (860 nmol). Data shown are mean \pm sem for pooled responses from n=5 rats. ** $P < 0.01$, * $P < 0.05$; unpaired t-test.

Capsaicin had a more rapid onset and a greater discharge frequency when compared to anandamide.

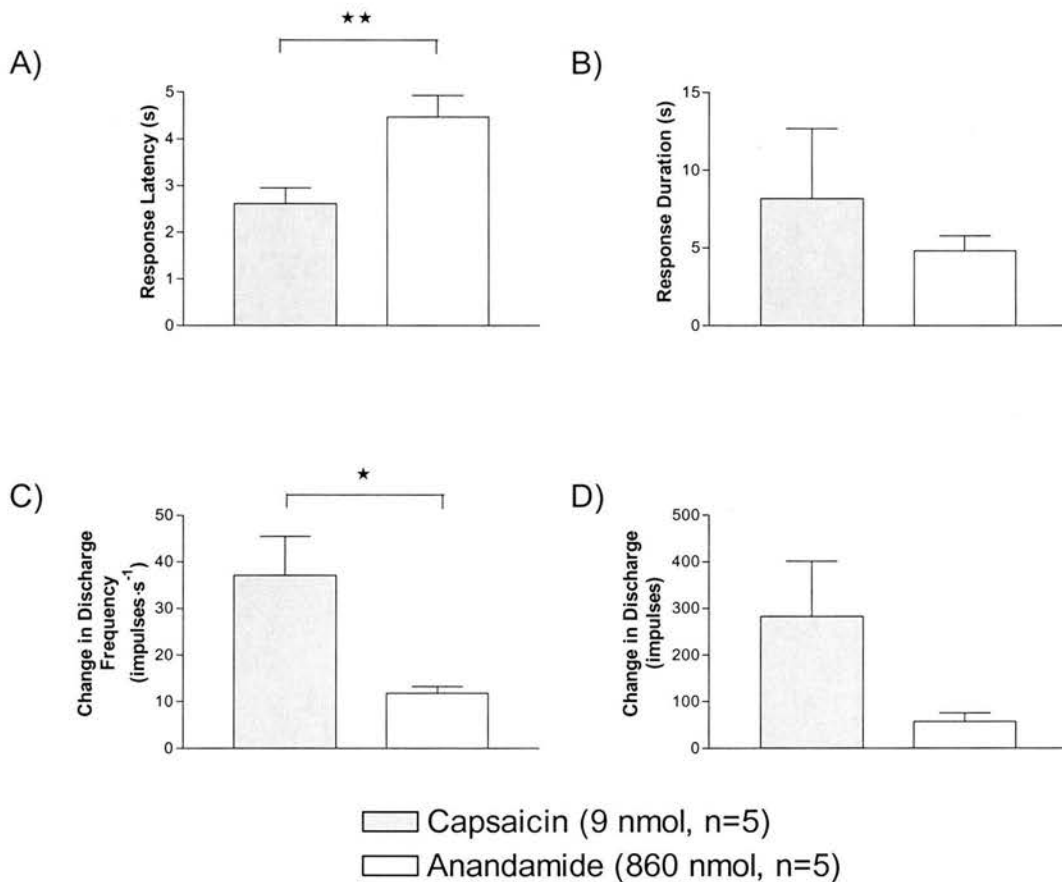
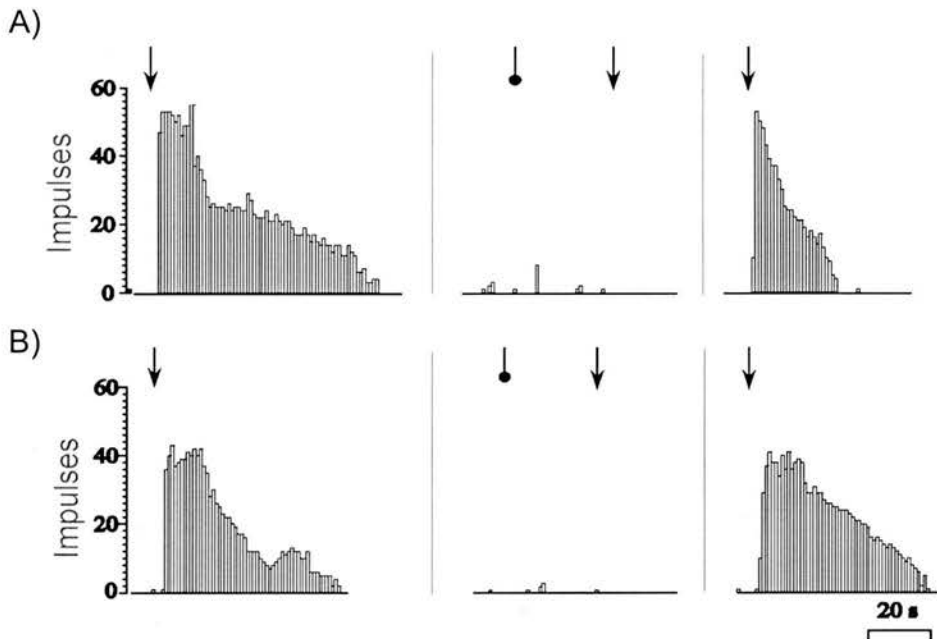


Figure 5.7 – Effect of capsazepine on afferent discharge evoked by capsaicin and anandamide from nociceptive afferents innervating a normal rat knee joint.

Afferent discharge from a single C-fibre innervating a normal knee joint before by A) capsaicin (9 nmol) or B) anandamide (2900 nmol) before and after a bolus injection of capsazepine 1mg kg^{-1} . Capsaicin was administered at the arrows and capsazepine at the circle. The time interval between panels is 20 minutes. Histogram shows the number of action potentials generated and is grouped into 1s bins.

Capsazepine reversibly abolished the excitatory responses to capsaicin and to anandamide.



6. FCA-induced unilateral arthritis in the mouse knee joint

6.1 INTRODUCTION

Experimental arthritis using FCA induces a chronic inflammation in the rat knee joint that causes sensitisation of sensory nociceptors. The unilateral nature of the inflammation allows the behavioural examination of hyperalgesia and allodynia associated with the inflammation. A number of inflammatory mediators have been examined in the rat including bradykinin (Kelly *et al.*, 1995a), 5-hydroxytryptamine (Birrell *et al.*, 1990a), ATP (Dowd *et al.*, 1998b) and adenosine (Dowd *et al.*, 1998a) using both behavioural testing of hyperalgesia and electrophysiological examination of nociceptor function. To fully characterise the responses requires specific agonists and antagonists for the receptor of interest. Developments in molecular biology now mean that receptors can be targeted by genetic manipulation without the need for specific pharmacological tools.

The manipulation of DNA in experimental animals, particularly in the mouse, has allowed nociception to begin to be examined at the level of the gene. Using this technology a great number of transgenic mouse lines have been derived with relevance to inflammatory or painful conditions in which a specific receptor or protein has been over expressed or completely removed in the animal from the start of development. This provides an opportunity to study these animals for alterations in their ability to develop chronic inflammation and the sensitisation of peripheral nociceptors by the application of appropriate models to the genetically altered animals.

MURINE MODELS OF ARTHRITIS

Animal models do not provide an exact mimicry of any human arthritic disease, however they do provide insight into key aspects of the human condition and can be used to improve therapeutic treatment. Models follow from specific ideas involving the aetiology of the condition.

Collagen-induced arthritis (CIA) is based on the principle that arthritis (particularly RA) is a result of a loss of tolerance against a collagen specific auto-antigen (Holmdahl *et al.*, 1988; Holmdahl *et al.*, 1989; Trentham *et al.*, 1977). The CIA model is characterised by a polyarthritis and a pathogenesis that is in many ways similar to that seen in RA with characteristic changes including an intense synovitis with severe cartilage and bone erosion by a pannus-like tissue (Stuart *et al.*, 1985). Although the model allows for the examination of mediators involved in autoimmune cartilage and bone destruction it is only inducible in susceptible strains. The susceptible strains include DBA/1 and B10RIII mice, however even in these strains only 20-40% of the animals develop arthritis with CIA ((Myers *et al.*, 1995; Myers *et al.*, 1997).

Other models utilise a local T-cell mediated inflammation to produce a long lasting inflammation. Antigen-induced arthritis (AIA) is produced by the introduction of methylated bovine serum albumin (mBSA) into the knee joint after immunisation (Brackertz *et al.*, 1977b; Brackertz *et al.*, 1977c). The mBSA is not readily cleared from the joint space and remains in the joint inducing a chronic inflammation by a local immune response (van den Berg *et al.*, 1982; van den Berg *et al.*, 1984). Once the response to the antigen has been established it is possible to produce flare-up by systemic administration (Lens *et al.*, 1984). AIA is inducible in

a number of mouse strains however the severity of the arthritis varies greatly between strains with C57BL6 mice producing a maximal response (Brackertz *et al.*, 1977a; Williams *et al.*, 1993).

Producing a local inflammation by the introduction of xenobiotic material from either yeast or bacteria can also induce arthritis by the activation of complement. Zymosan is a yeast component that when injected into the knee joint of mice produces cartilage and joint destruction (Keystone *et al.*, 1977; van den Berg *et al.*, 1981). Streptococcal cell wall (SCW) induced arthritis, in addition to stimulating complement, also shows cross reactivity as activated immune cells attack components of the articular cartilage that resemble bacterial constituents. Given systemically in the rat SCW injection leads to a severe polyarthritis with a pathogenesis like that seen for systemic administration of FCA (van den Broek, 1989). In the mouse, systemic injection of SCW does not lead to a chronic inflammation however arthritis can be induced by either single or repeated local injections (van den Broek *et al.*, 1988).

AIA and SCW-arthritis are characterised by inflammatory cell infiltration, hyperplastic changes in the synovial lining as well as pannus formation with associated cartilage destruction and bone erosion (Andersson *et al.*, 1998a; Andersson *et al.*, 1998b; van den Broek *et al.*, 1988).

TRANSGENIC MOUSE STRAINS AND ARTHRITIS

Murine models of arthritis permit the examination of the role of a specific receptor or protein in the development and maintenance of the disease state by testing the model on transgenic animals. Genetically modified animals are theoretically capable of conclusively showing the relevance of a particular receptor

or mediator by eliminating it completely through disrupting the gene sequence or over-expressing it by adding multiple copies of the gene into the DNA of the animal. However, it should be noted that genetic manipulation does not always produce the expected phenotype; this observation is particularly common where genes encoding targets known to be involved in pain processing are deleted, producing no overt disease-relevant phenotype (COX-1, COX-2, mu-opiate – see (Wilson & Mogil, 2001)). In these cases, observation of the effect of the transgene would not have supported development of analgesics based on manipulation of these targets, yet clearly such analgesics are clinically effective. Nonetheless, in the absence of effective pharmacological tools, the transgenic process remains an important and potentially useful manner of assessing the effectiveness of manipulation of a target in models of disease. This process was used to examine the role of the purinoceptor P2X₇ in experimental arthritis by producing a strain of mice lacking the P2X₇ receptor.

The P2X₇ receptor is a subtype of ATP-gated cation channel receptors (Khakh *et al.*, 2001; Rassendren *et al.*, 1997) that are expressed primarily on immune cells (Chessell *et al.*, 1997; Collo *et al.*, 1997; DiVirgilio, 1995; DiVirgilio *et al.*, 1998; Ferrari *et al.*, 1997b). As extracellular levels of ATP are elevated during inflammation it has been proposed that the P2X₇ receptor may play a role in the development of inflammatory conditions. ATP is able to trigger the release of the pro-inflammatory cytokine IL-1 β from immune cells and it does this via activation of the P2X₇ receptor (Ferrari *et al.*, 1997a; Grahames *et al.*, 1999). Once released, IL-1 β has been shown to be responsible for cartilage degradation and bone erosion in a number of animal models (Joosten *et al.*, 1999; Kuiper *et al.*, 1998) and in human

RA patients (van den Berg, 2000a). Direct injection of IL-1 into the knee joint has been shown to produce arthritis in rabbits (Pettipher *et al.*, 1988), rats (Hrubey *et al.*, 1991) and mice (Chandrasekhar *et al.*, 1990; van de Loo *et al.*, 1989) and an existing inflammatory condition can be exacerbated by IL-1 injection (van de Loo *et al.*, 1992). Furthermore, studies with monoclonal antibodies have shown that blocking IL-1 reduces arthritic changes (Joosten *et al.*, 1999; Joosten *et al.*, 1996). If the P2X₇ mediated release of IL-1 contributes to a large proportion of the IL-1 present in arthritic joints then the interruption of receptor function may go a long way to preventing the pathological changes seen in arthritis.

AIMS OF THE STUDY

The aim of this study was to use a unilateral model of murine arthritis to examine transgenic mice with alterations relevant to chronic inflammation and pain. We examined previously described methods for the induction of unilateral arthritis in mice. When it was shown that existing methods did not provide the model required we developed a new model using intra-articular injections of FCA. The model was characterised to show its relevance as an arthritis-like inflammation. The model was then used to examine the role of the purinoceptor P2X₇ in the induction or experimental arthritis. Furthermore, to correlate with electrophysiological work done in the rat, a technique was developed to permit recording from sensory afferents innervating the mouse knee joint.

6.2 METHODS

INDUCTION OF UNILATERAL ARTHRITIS

This component of the thesis details the development of a murine model of unilateral arthritis. The protocol presented below is the final adopted method for the induction of chronic inflammation.

Male C57 Black 6 (C57BL6; Charles River, UK) and DBA-1 (Harlan, UK) mice (8 weeks old) were used to establish a unilateral model of chronic joint inflammation. Animals were housed 8 per cage in a 12-hour light/dark environment and were given free access to standard animal feed and water for the duration of the study. Mice were injected with either Freund's Complete Adjuvant (FCA, 1 mg ml⁻¹ *Mycobacterium tuberculosis* in paraffin oil, Sigma) or vehicle (heavy liquid paraffin oil, HLP). Briefly, injections were carried out during transient anaesthesia (3% halothane in oxygen) and a small incision was made over the left knee joint to allow visual identification of the patella tendon. FCA or HLP (5 µl) was injected under the patella tendon and directly into the synovial space of the knee joint using a 30-gauge needle mounted on a 50 µl Hamilton syringe. To achieve a long lasting inflammation the injections were repeated 4 times (once a week for four weeks).

ASSESSMENT OF ARTHRITIS

Animals were scored for inflammation and a hyperalgesic or allodynic response to a mild noxious stimulus, and weighed; this was done before inducing arthritis, and at least twice weekly thereafter. Joint swelling, gait and hyperalgesia were each measured subjectively (scored 0-3, where 3 is maximal). The same

experienced operator performed all measurements and used his experience to apply consistent stimuli that were subjectively assessed.

Body Weight

Body weight of the animals was monitored throughout the experiment and was used as an overall measure of animal health and potential level of discomfort associated with the repeated injection protocol. Animals in severe chronic pain will not gain weight as normal (Greenwald & Diamond, 1988).

Inflammation

Inflammation (when present) induced by either FCA or HLP was monitored throughout the experiment by measuring the joint diameter of both the injected and uninjected hind limb using micro-callipers, accurate to 0.01 mm (Mitutoyo, Japan). Joint swelling was also assessed subjectively by evaluation using a 0 (normal) to 3 (extreme joint deformation).

Hyperalgesia/weight bearing allodynia

Subjective measures were used to correlate apparent hyperalgesia and allodynia with inflammation of the joint induced by FCA; a 0 (normal) – 3 (extreme discomfort) scale of the response to a noxious stimulus was employed in the absence of any rapid, reproducible and convenient qualitative index. Testing involved squeezing the joint between thumb and forefinger and determining the level at which the animal withdrew the limb (normal end-point) or vocalised (rare). To estimate the amount of pressure being applied to the joint, saline-filled elastic tubing the same diameter as the joint and connected to a pressure transducer was squeezed in a

similar manner to the joint to provide a force in pressure in g/mm^2 (Table 6.2). In the majority of cases, in response to gradually increasing applied pressure the animal withdrew the limb without vocalising. Similarly the weight-bearing allodynia (favouring of the injected limb during normal locomotion) was assessed by examining the gait of the animal and protecting the injected limb by raising it off the ground, again qualitatively by using a 0 (normal gait) to 3 (three legged gait) scale.

Drug treatment

Treatment of an established arthritis was carried out using two standard drugs, namely the steroid prednisolone and the non-steroidal anti-inflammatory indomethacin and vehicle-treated controls. Prednisolone (1 mg kg^{-1}) was administered daily by intra-peritoneal (i.p.) injection. Indomethacin (1 mg kg^{-1}) was administered daily by sub-cutaneous (s.c.) injection. Treatment began 7 days after the final injection of FCA on experimental day 28 and was continued for a period of 7 days. Drugs were coded before the injections began and were unknown to the person measuring the animals until the end of the experiment.

Histopathology

Animals were killed on day 28 (no drug treatment) or day 35 (with drug treatment) by cervical dislocation and the left and right knee joints surgically removed for histology. In some cases the ipsilateral ankle joint was removed to check for spread of the inflammation to additional joints.

Fixation of tissue

The knee joints were collected by cutting through both the femur and the tibia. Joints were taken with the skin intact and placed into screw-top vials where they were fixed in 30 ml of 10 % neutral buffered formalin (Sigma) for a minimum of 5 days at room temperature.

Decalcification of knee joints

Fixed tissue was transferred from the vials to cassettes and then decalcified in Kristenson's Fluid (5.1 M formic acid, 0.6 M sodium formate) for 1 week. After 1 week the samples were trimmed to remove excess tissue. The skin was removed from the samples and then a cut was made straight through the joint just off the midline. Samples were placed cut side down in the cassettes and returned to the decalcification solution for at least one week.

Histology and staining

After decalcification the samples were embedded in paraffin wax and sectioned. Briefly, samples were stored on ice until they were sectioned to keep the tissue and wax firm. Two sections, each of 3 μ m, were cut from each joint and floated out in a 50 °C water bath. Each section was transferred to a labelled glass slide. For each knee joint one section was stained with haematoxylin/eosin and the other section stained with toluidine blue.

Evaluation of joint damage

The stained sections were evaluated at GlaxoSmithKline (Ware, UK) by Dr. Christopher Clarke. The evaluation was done on coded specimens without prior knowledge of the treatment groups or transgenic status. The severity of the

inflammation, joint damage, and characteristic markers of arthritis was arbitrarily scored subjectively on a sliding scale (1 = slight to maximum 5 = very marked) in the categories shown in Table 6.1.

Electrophysiological recording from the saphenous nerve

Male C57BL6 mice (8-12 weeks), either normal or 7-14 days after treatment with FCA, were used for the electrophysiological characterisation of sensory afferents innervating the knee joint. The preparation was set up much the same as for the rat (see Chapter 2.2) with a few changes made due to size differences between rats and mice – for a schematic of the preparation see Figure 6.1. The small size of the nerve made it impossible to record from the medial articular nerve (MAN) in the mouse. The afferents that make up the MAN join the main saphenous nerve in the medial aspect of the leg, so recordings were made from the saphenous nerve and the input restricted to afferents that make up the receptive field of the knee joint by eliminating all responses other than that through the MAN.

Anaesthesia and surgical procedures

Animals were anaesthetised with urethane (0.7 ml per 100 g of body weight, i.p., 20% w/v ethyl carbamate in saline). Deep anaesthesia was defined as the point at which the toe-pinch withdrawal reflex was abolished. If the initial injection of urethane did not produce deep anaesthesia then additional urethane (0.1 ml per injection; i.p.) was given until the mouse was adequately anaesthetised. The animal was placed on a thermal blanket to maintain body temperature at 37°C. The trachea was cannulated (cannula OD: 1.0 mm) to facilitate artificial respiration if required. The right femoral artery was cannulated with a small diameter cannula (OD < 0.5

mm), made by pulling out a plastic 1 ml syringe. The cannula was inserted as near as possible to the abdominal aorta to allow for the introduction of drugs to the left hind limb by close arterial injection (i. art.) - as in the rat (see Chapter 2.2)

Recording from the saphenous nerve

The left hind limb was fixed to a stable support and a small pouch made by opening the skin above the joint and sewing it to a small brass ring. The pouch was filled with HLP to produce an electrically isolated system. Recording from the very fine afferents innervating the mouse knee joint involved cutting the saphenous nerve above and below the knee, thereby recording only from afferents with receptive fields in the knee joint. Monopolar recording of afferent neural discharge from nerves innervating the joint was recorded using bipolar platinum-iridium electrodes where the saphenous nerve was laid on one electrode while the other electrode was grounded via fatty tissue surrounding the joint. Impulses were recorded and analysed as for neural recordings in the rat (see section 2.2).

6.3 RESULTS

INDUCTION OF UNILATERAL ARTHRITIS IN MICE

Sub-cutaneous injection of FCA around the mouse ankle joint

DBA/1 mice were originally used because of their susceptibility to arthritic conditions - both spontaneously occurring and artificially induced (see Introduction). Initially the hypothesis tested was that unilateral arthritis could be induced in mice, by a single sub-cutaneous injection of FCA over the ankle joint, as in rats (Birrell *et al.*, 1990a; Birrell *et al.*, 1990b; Birrell *et al.*, 1991; Birrell *et al.*, 1990c; Grubb *et al.*,

1991; Grubb *et al.*, 1988; McQueen *et al.*, 1990). Furthermore, because of the difference in size between rats and mice, the mouse ankle provided easier access for the purposes of measuring the joint diameter. A single subcutaneous injection of FCA (50 µg) over the ankle joint failed to induce chronic inflammation in DBA/1 mice (Figure 6.2), although there was an initial inflammation that returned to near basal levels after approximately 7 days. Unlike the response in the rat, there was no secondary chronic phase, and indeed at 21 days there was no significant difference in the diameter of the injected joints in the FCA treated group and those of either the contralateral limb, the ipsilateral limb in uninjected control animals, or animals that received the HLP control.

Sub-cutaneous injection of FCA around the mouse knee joint

The aim was to establish a chronic inflammation in the knee joint of the mouse to permit pharmacological studies on nociceptive afferents, as in our existing rat model (Dowd *et al.*, 1998a; Dowd *et al.*, 1998b). Therefore all further development was done in the knee joint rather than first establishing the model in the ankle and subsequently transferring it. An attempt was made to induce a chronic inflammation by a single sub-cutaneous injection of FCA over the knee using a larger dose than that used in the ankle. The results show that FCA given at either 100 or 150 µg was unable to induce chronic inflammation (Figure 6.3). There was no significant difference between either dose of FCA and HLP controls at day 22 ($P = 0.5320$, Kruskal-Wallis), or between the injected and uninjected limbs in the FCA treated groups at either dose ($P = 0.53$, $P = 0.42$ respectively; paired t-test). Furthermore, in animals treated with 150 µg of FCA on day 0, followed by a booster

injection of FCA (100 µg, s.c. over the knee) at day 22, there was still no chronic inflammation, and the diameter of the injected joint did not differ significantly that of HLP controls ($P = 0.42$, day 45, Mann-Whitney).

Antigen induced arthritis with methylated bovine serum albumin

As FCA injected around the joint failed to cause chronic inflammation, it was decided to administer FCA by intra-articular injection into the mouse knee joint. A number of days were spent learning the technique from Dr. Leo Joosten in the Department of Rheumatology, University of Nijmegen, The Netherlands. The group at the University of Nijmegen had developed a number of unilateral models of murine arthritis, including an antigen-induced model of arthritis (AIA), as detailed in the introduction. Upon returning to Edinburgh the AIA model in C57BL6 mice using methylated Bovine Serum Albumin (mBSA) was investigated. Briefly the technique involved immunising each animal on day 0 with 100 µg of mBSA in 100 µl of PBS/FCA (1:1), injected sub-cutaneously at six different points on the back of the animal (~17 µl/injection). On day 7 the animals were given a booster of 100 µg mBSA in 100 µl of PBS/FCA (1:1), injected at two sites on the back. Finally on day 28 the animals were challenged with 60 µg of mBSA in 6 µl saline injected i. art. into the left knee joint under transient anaesthesia (3% halothane in oxygen). Control animals were injected with saline only. Animals were expected to show a maximum response 4-5 days after the challenge, and a sustained inflammation that would still be present 28 days post-challenge (van den Berg *et al.*, 1984). The results obtained (Figure 6.4) showed that in C57BL6 mice an inflammatory response occurred, which peaked at day 5; however this inflammation resolved back to basal

levels within 14 days of the challenge. A similar result was seen in the DBA/1 mice (Figure 6.5), which also failed to exhibit chronic inflammation.

Unilateral arthritis with repeated injections of FCA in DBA/1 mice

According to Dr. Joosten the method utilising purified streptococcal cell walls (SCW) was robust and had worked in every strain of mice that they had tested (personal communication). In addition it was anticipated that repeated injections of SCW would generate a chronic inflammation. As I had previously shown that FCA could induce inflammation of joints in the mouse, the hypothesis that a chronic inflammation should be obtained using the bacterial components present in the FCA, in the same way as was possible with the SCW model, was tested.

Knee joint diameter

It proved possible to induce a chronic inflammation of the knee joint with repeated injections of FCA in DBA-1 mice. Subjective examination of the mice throughout the experiment showed that all animals (8 of 8) treated with FCA showed some form of inflammation, and in every mouse the inflammation was restricted to the injected left knee joint. There was no evidence of inflammation present in the contralateral limb (Figure 6.6). Prior to the injections of FCA (day 0) there was no significant difference between the two groups in the diameter of the left knee joint ($P = 0.33$; unpaired t-test). Furthermore in the group that was to receive the FCA there was no significant difference between the left and the right knee joint ($P = 0.61$; paired t-test). The group that was to receive HLP did show a slight difference in the starting diameters between the left and right knee joints that was just statistically significant ($P = 0.05$; paired t-test).

After each injection of FCA (5 μ l, i.art.) there was an acute inflammation that resolved, as had been the case seen with the single sub-cutaneous injections. With each subsequent injection the acute phase of the inflammation, as measured by the diameter of the knee joint, was significantly greater. In addition, as the acute inflammation resolved the diameter of the knee joint never returned to its starting size, indicating residual alterations. After four injections, the joint size had increased markedly and the accumulated changes were consistent with chronic inflammation. The cumulative effect of multiple challenges is shown schematically in Figure 6.7. At day 28 the diameter of the left (injected) knee joint was significantly increased in the group treated with FCA when compared to HLP controls ($P = 0.005$; unpaired t-test; Figure 6.6A). This increase in joint diameter was restricted to the injected joint as there was no significant difference in the contralateral knee joint between FCA and HLP treated mice at day 28 ($P = 0.07$; Mann-Whitney; Figure 6.6B). As the inflammation was restricted to the left (injected) knee joint we were able to use the right knee as an internal control and examine the percent increase in the injected joint (left) when compared to the uninjected (right) joint (Figure 6.6C). The percent increase in the diameter of the injected joint was greater in FCA treated animals ($16.7 \pm 2.7\%$) when compared to HLP controls ($2.9 \pm 1.0\%$; $P = 0.001$, paired t-test). Examination of the knee joint at day 28 in comparison to the starting diameter at day 0 (Figure 6.6D) again also showed that there was an increase in the FCA injected joint (0.69 ± 0.13 mm) that was not seen in the HLP controls (0.25 ± 0.04 mm) and that this increase was statistically significant ($P = 0.007$; unpaired t-test).

Body weight

The level of discomfort induced in these animals was sufficient in some cases to produce limping in the days immediately following each injection. However the animals continued to feed normally and there was no significant difference in their weight gain when compared to HLP-injected control animals. Throughout the course of the experiment there was no significant difference in the average body weight of the two groups (day 0: $P = 0.38$, day 14: $P = 0.34$, day 28: 0.20; unpaired t-tests; Figure 6.8A). This was further confirmed when reviewing weight gain in individual animals by looking at the change from the starting body weight at day 0 (Figure 6.8B). There was no significant difference ($P = 0.48$; unpaired t-test) in the change in body weight between FCA treated (3.0 ± 0.2 g) and HLP treated (3.3 ± 0.3 g) groups at day 28.

Hyperalgesia/allodynia

After each injection of FCA there was a short period during which some of the animals displayed discomfort in walking, as manifest by a three-legged gait (measured as 3 on the 0-3 scale, see section 6.2). This was observed in both the FCA and the HLP treated groups and was therefore probably a consequence of mild trauma associated with the injection procedure.

Discomfort in walking during chronic inflammation

Seven days after the final injection, the acute inflammation associated with the last injection resolved and the animals were left with a chronic arthritis in the injected knee joint. Subjective examination of the animals showed that, in mice treated with repeated injections of FCA, only 2/8 (25%) animals in the FCA group and 0/8 (0%) animals in the HLP treated group exhibited a mild limping behaviour,

scored as 1 on the subjective 0-3 scale (Figure 6.9). The majority of animals showed normal gait even when extensive inflammation was present in the knee joint.

Response to noxious pressure on the knee joint

The withdrawal response to a noxious stimulus (squeezing the joint at an increasing intensity until the animal withdrew its limb) was also measured in order to establish the degree of hyperalgesia associated with the experimental inflammation. Animals receiving repeated intra-articular injections of FCA showed a decrease in the threshold pressure evoking withdrawal at day 28, seven days after the final injection, when compared with the contra-lateral joint ($P = 0.004$; Mann-Whitney) or the vehicle treated controls ($P = 0.004$; Mann-Whitney) – see Figure 6.10A. In FCA treated animals at day 28 the majority of mice, 7/8 (87%), showed some sensitisation to moderate pressure applied to the joint (median hyperalgesia score: 1.5) that was not seen in the contralateral limb (median: 0) or in HLP controls (median: 0) in which no animals (0/8 mice for each; 0%) displayed hyperalgesia. Furthermore the hyperalgesia score was significantly correlated ($r^2 = 0.1724$; $P = 0.003$, Spearman Rank Correlation; Figure 6.10B) with the increase in the diameter of the joint, indicating that the degree of swelling present in the joint was directly related to the hyperalgesia experienced by the animal.

Histopathology

Knee joints were collected from recently killed animals from all groups on day 28, 7 days after the last injection of FCA or HLP. The joints were processed as stated in the methods and midline sections were stained either with haematoxylin and eosin (H&E) or Toluidine Blue. Samples were coded and scored blind.

Figure 6.11 shows the pathological changes resulting from repeated injections of FCA or HLP into the mouse knee joint. The right knee joint (uninjected) showed no notable changes. All FCA treated animals showed pathological changes in the left knee joint (injected). There was moderate hypertrophy of the synovial membrane and the infiltration of a large number of PMNs and macrophages into the joint space. In all animals there was extensive inflammation of tissues outside the joint including tendons, ligaments and surrounding muscle. Small amounts of pannus were noted producing mild erosion of the articular cartilage and slight changes in bone formation and/or erosion in most animals. Thickening of the synovial membrane, PMN infiltration and peri-articular inflammation were seen in animals receiving HLP, however far less severe than that seen in arthritic joints.

Pathological changes were graded on a 0 (normal) to 5 (severe) scale (Table 6.1) by Dr. Christopher Clarke (GlaxoSmithKline, Ware). Gradings were pooled as a histological score for each individual knee joint (Figure 6.12). There was a significant difference in the histological score between the left (injected) and right (uninjected) knee joint in both FCA and HLP treated animals ($P = 0.0006$ and $P = 0.0006$, respectively; Mann-Whitney). The median histological score in FCA injected joints was 12 ($n=8$) and 5 ($n=8$) for HLP injected joints. This difference was found to be significant ($P = 0.002$; Mann-Whitney)

Unilateral arthritis with repeated injections of FCA in C57BL6 mice

In order to develop a robust model of unilateral arthritis it was necessary to induce the inflammation in more than one strain of mouse, as there can be large strain difference in experimental arthritis models. The transgenic animals that we were planning to study have a C57BL6 genetic background, and consequently

C57BL6 animals were used. Experiments were performed on 8-week-old mice with 8 mice per group.

Knee joint diameter

As with the DBA/1 mice, subjective examination over the course of the experiment showed that it was possible to induce a chronic inflammation in the knee joint of adult male C57BL6 mice with repeated injections of FCA (Figure 6.13). All mice in the FCA treated group (8/8; 100%) showed moderate to severe inflammation in the injected joint compared to only 1/8 (12%) mice showing a mild inflammation in vehicle controls. Prior to the injection of either FCA or HLP there was no significant difference between groups in the diameter of either the left or right knee joints ($P = 0.51$ and $P = 0.12$ respectively; unpaired t-test). One week after the repeated injections of FCA (on day 0, 7, 14, and 21) there was a significant increase in the average diameter of the left (injected) joint when compared to either the contralateral (uninjected) limb ($P = 0.0001$; paired t-test) or the corresponding limb injected with HLP in the control animals ($P = 0.0001$; unpaired t-test; Figure 6.13A). This increase in diameter was restricted to the injected limb - there was no significant difference between the right (uninjected) limb and either limb in the HLP controls ($P = 0.12$ and 0.25 respectively; unpaired t-test; Figure 6.13B). As there was no change in the right limb it was possible to use it as an internal control and express the increase in diameter of the left limb as a percentage of the right limb's diameter for each animal (Figure 6.13C). On average, on day 28 there was an increase in diameter of the left knee in comparison with the right knee diameter of FCA treated animals ($21.2 \pm 2.0\%$). That did not occur in HLP treated controls ($2.5 \pm 1.1\%$). This increase was statistically significant ($P = 0.0001$; unpaired t-test). The diameter of

the knee joint at day 28 was also compared to the diameter pre-FCA (Figure 6.13D); there was an increase in joint diameter of FCA treated mice ($+0.97 \pm 0.11$ mm) that was not seen in the HLP controls (-0.03 ± 0.05 mm) which was statistically significant ($P = 0.0001$; unpaired t-test).

Body weight

Body weight was used as an overall measure of animal health and potential level of discomfort associated with the repeated injection protocol (Figure 6.14). Prior to injection with either FCA or HLP there was no significant difference in the average weight between the two groups ($P = 0.23$; unpaired t-test). Both FCA and HLP treated groups gained weight at similar rates and at day 28, 7 days after the final injection; there was no significant difference in the average body weight between the two groups ($P = 0.65$; unpaired t-test: Figure 6.14A). In addition, on average, there was no difference ($P = 0.20$; unpaired t-test) in the weight gained by each animal at day 28 when compared to the starting weight at day 0 for either FCA (3.3 ± 0.4 g) or HLP (2.5 ± 0.4 g) – see Figure 6.14B.

Hyperalgesia/allodynia

As stated above there was a period (3-4 days) following each injection of either FCA or HLP in which some animals displayed discomfort in walking as manifest by limping, or in extremis, a three-legged gait. As it was present in both groups it was likely to have been associated with the mild trauma resulting from the injection procedure.

Discomfort in walking during chronic inflammation

Seven days following the final injection of FCA at day 28, the acute inflammatory response had resolved, and as shown above, some animals were left with a chronic inflammation present in the left knee joint. Subjective examination showed that a number of animals in the FCA group (3/8; 38%) showed discomfort in walking whereas there were no animals showing such behaviour in HLP control group (0/8; 0%) – See Figure 6.15A. However the discomfort in walking observed was very mild and was scored as a 1 on the 0-3 scale (see section 6.2). There was, however, was a significant correlation ($P = 0.004$; $r^2 = 0.48$; Spearman Rank Correlation) between the discomfort in walking and the level of inflammation present in the joint (Figure 6.15B).

Response to noxious pressure on the knee joint

The withdrawal response evoked by a mild noxious stimulus (squeezing of the joint) was measured to determine the degree of inflammation-associated hyperalgesia. Animals receiving repeated intra-articular injections of FCA showed an increase in the withdrawal response at day 28 when compared to the contralateral joint ($P = 0.0002$; Mann-Whitney) or the vehicle treated controls ($P = 0.002$; Mann-Whitney) – see Figure 6.16A. All FCA treated animals (8/8, 100%) showed some form of sensitisation to squeezing the injected joint (median hyperalgesia score: 2) that was not seen in the contralateral joint (median hyperalgesia score: 0; $n = 8$) or the corresponding limb in HLP controls (median hyperalgesia score: 0.5; $n = 8$). Furthermore, the amount of inflammation present in the injected knee joint at day 28

significantly correlated with the hyperalgesia score ($P = 0.0001$; $r^2 = 0.70$; Figure 6.16B).

Histopathology

Knee joints were collected from recently killed animals from all groups on day 28, 7 days after the last injection of FCA or HLP. The joints were processed as stated in the methods and midline sections were stained either with haematoxylin and eosin (H&E) or Toluidine Blue. Samples were coded and scored blind.

Figure 6.17 shows the pathological changes resulting from repeated injections of FCA or HLP into the mouse knee joint. The right knee joint (uninjected) showed no pathology. All animals that received repeated intra-articular injections of FCA showed pathological changes consistent with a moderate arthritis. These changes were restricted to the left knee joint. In all animals there was extensive peri-articular inflammation affecting the tendons, ligaments and surrounding muscle. There was moderate hypertrophy of the synovial membrane and the infiltration of a large number of PMN and macrophages into the joint space. Small amounts of pannus were noted producing mild erosion of the articular cartilage, however formation and/or erosion of the bone surface was present only in two animals. Thickening of the synovial membrane, PMN infiltration and peri-articular inflammation were seen in animals receiving HLP, however far less severe than that seen in arthritic joints.

Pathological changes were graded on a 0 (normal) to 5 (severe) scale (Table 6.1) by Dr. Christopher Clarke (GlaxoSmithKline, Ware). Gradings were pooled as a histological score for each individual knee joint (Figure 6.18). There was a significant difference in the histological score between the left (injected) and right (uninjected) knee joint in both FCA and HLP treated animals ($P = 0.0006$ and $P =$

0.01, respectively; Mann-Whitney). There was a significant difference in the histological score between FCA (median: 13; n=7) and HLP injected joints (median: 5; n=4; $P = 0.006$; Mann-Whitney).

COMPARISON OF UNILATERAL ARTHRITIS DEVELOPMENT IN DIFFERENT STRAINS OF MICE

As detailed in the introduction many existing models of arthritis in mice display large variation when transferred from one strain of mouse to another (Brackertz *et al.*, 1977a; Wooley *et al.*, 1981). It was therefore necessary to examine the differences between development of RA in the two strains being investigated, namely DBA/1 and C57BL6. As the two strains vary in size when adult, all the results were compared in terms of a percentage differences in adult mice.

Knee joint diameter

The percent increase in the left knee diameter in comparison to the right was examined in both DBA/1 and C57BL6 mice. Prior to the injection of FCA there was no significant difference between the two strains ($P = 0.22$; unpaired t-test). Once chronic inflammation was induced (day 28) there was a significant increase in the left knee diameter when compared to the right in each strain with a $16.7 \pm 2.7 \%$ increase in DBA/1 mice and $21.2 \pm 2.0 \%$ in C57BL6 mice. Comparing the two strains there was no significant difference in these increases ($P = 0.21$; unpaired t-test; Figure 6.19).

Hyperalgesia/allodynia

Discomfort in walking in chronic inflammation

Once chronic inflammation was induced, there was no effect on mobility in these animals. The median walking score for both groups at day 28 was 0, and both strains seemed to adapt equally well to the chronic inflammation (Figure 6.20).

Response to noxious pressure on the knee joint

Both C57BL6 and DBA/1 strains showed sensitisation to noxious pressure (median scores: 1.5 and 2 respectively) of the injected knee joint, but not of the contralateral knee (Figure 6.21). There was no significant difference between DBA/1 and C57BL6 mice in their response to applied noxious pressure ($P = 0.09$; Mann-Whitney).

Histopathology

The pathological changes induced by repeated injections of FCA were broadly similar in both DBA/1 and C57BL6 mice – see Figures 6.11 and 6.17. Changes seen in both strains included inflammation of the peri-articular tissues, infiltration of the joint space by PMNs and macrophages, and thickening of the synovial membrane. In most cases there was mild pannus formation that resulted in damage to articular cartilage and minor bone changes.

Comparing the histological scores showed that there was no significant difference between DBA/1 and C57BL6 in terms of the overall severity of the pathological changes induced by the intra-articular injections of FCA ($P = 0.57$; Mann-Whitney; Figure 6.22).

EFFECT OF THE STEROID PREDNISOLONE ON ESTABLISHED FCA-INDUCED UNILATERAL ARTHRITIS DBA/1 MICE

To validate the model of FCA-induced unilateral arthritis it was necessary to establish the effects of a number of “gold standards” in terms of inflammation and pain relief. One of the drugs we used was the steroid, prednisolone, commonly used in man as an effective anti-inflammatory (Kelley *et al.*, 1997). The experiment was carried out in two groups of 8 week-old, male DBA/1 mice (n=8 per group). Unilateral arthritis was induced in the left knee joint as detailed above. Animals were monitored both subjectively and quantitatively throughout the experiment to determine the extent of the inflammation and hyperalgesia/allodynia produced. Measurements were made twice weekly, once just prior to each injection of FCA and once midway between each injection. After unilateral arthritis was established the mice continued to be measured during treatment with either prednisolone (10 mg kg⁻¹ i.p. daily) or vehicle. Drugs were coded and administered blind.

Knee joint diameter

The diameter of both the left (injected) and right (uninjected) knee joints was monitored throughout the course of the experiment. Chronic unilateral arthritis was induced in both groups and treatment with prednisolone or vehicle began on day 28, seven days after the final injection of FCA (Figure 6.23). Prior to the injection of FCA (day 0) there was no significant difference in the diameters of either left or right knee joints ($P = 0.64$ and $P = 0.76$ respectively; unpaired t-tests).

Pre-treatment: After the repeated injections of FCA (day 28) there was no significant difference between the two groups in the diameter of either knee joint

(left: $P = 0.71$, Right: 0.16 ; unpaired t-test). There was a significant difference in both groups when comparing the left and right knee diameters ($P = 0.79$ and $P = 0.37$; paired t-test). The extent of the inflammation induced was similar for both groups. The percent increase in the left knee diameter when compared to the right knee was $11.3 \pm 1.8 \%$ and $12.6 \pm 1.9 \%$, which were not statistically different ($P = 0.62$; unpaired t-test).

Response to treatment: At day 28 treatment with daily i.p. injections of prednisolone (10 mg kg^{-1}) or vehicle for seven days commenced. After the seven days of treatment, at day 35, there was a significant decrease from values on day 28 in the left to right knee diameter ratio of prednisolone treated mice ($-10.3 \pm 2.9 \%$; $P = 0.002$; paired t-test) that was not seen in the vehicle controls ($-0.4 \pm 1.8 \%$; $P = 0.10$; paired t-test; Figure 6.23C). Furthermore examining each animal for the change in the left knee diameter from day 0 showed that on average prednisolone treated animals had returned to baseline levels (pre-FCA treatment) with a mean change of $+0.02 \pm 0.04 \text{ mm}$ from the starting diameter, which was significantly different ($P = 0.0001$; unpaired t-test) in comparison with vehicle controls, which remained chronically inflamed ($+0.43 \text{ mm} \pm 0.06 \text{ mm}$) with respect to day 0 values (Figure 6.23D).

Body weight

There was no significant difference between the two groups in mean body weights, either prior to FCA treatment ($P = 0.21$; unpaired t-test), or once the chronic inflammation had been established at day 28 ($P = 0.39$; unpaired t-test; Figure 6.24).

Pre-treatment: In addition, on average there was no significant difference between the two groups in the weight gained by each animal up to day 28 (3.0 ± 0.2 g and 3.4 ± 0.1 g; $P = 0.10$; unpaired t-test).

Response to treatment: Seven days of treatment with prednisolone induced a weight loss in the treated group, as there was a significant difference in average body weight at day 35 in comparison with vehicle controls ($P = 0.02$; unpaired t-test; Figure 6.24A). Examining the body weight over the entire course of the experiment the change from day 0 to day 35 was not statistically significant when comparing prednisolone and vehicle treated groups (2.6 ± 0.3 g and 3.3 ± 0.2 g respectively; $P = 0.09$; unpaired t-test; Figure 6.24B).

Hyperalgesia/allodynia

Discomfort from walking during chronic inflammation

Pre-treatment: All animals displayed evidence of chronic inflammation on day 28, but neither group showed any sign of difficulty in walking. Prior to prednisolone or vehicle treatment each group contained individual animals displaying mild limping behaviour (median score: 0 and 1 for 2/8 mice and 6/8 mice respectively Figure 6.25A).

Response to treatment: After seven days of treatment with prednisolone there was no sign of discomfort as the steroid-treated animals walked normally. In the vehicle treated group, where 6 animals had shown mild limping at day 28, limping was seen in only one animal (Figure 6.25B).

Response to noxious pressure on the knee joint

Pre-treatment: Immediately prior to treatment with prednisolone or vehicle, at day 28, both groups were sensitive to a painful stimulus as a result of chronic inflammation. This was restricted to the left joint (median scores: 1.5 and 2 in 8/8 for both groups), as there was no sensitisation in either of the right knee joints (median score: 0 in 8 mice in both groups). The difference between the left knee median scores was not significant ($P = 0.22$; Mann-Whitney; Figure 6.26A).

Response to treatment: After seven days of treatment with either prednisolone or vehicle, at day 35, the prednisolone treated group showed a reduced sensitivity/increased threshold to a noxious pressure stimulus applied to the joint (median score: 0) that was not seen in the vehicle controls (median score: 1.5) – see Figure 6.26B. The difference was statistically significant ($P = 0.007$, Mann-Whitney).

Histopathology

Knee joints were collected from recently killed animals from all groups on day 35 following 7 days of treatment with either prednisolone (10 mg kg^{-1}) or vehicle. The joints were processed as stated in the methods and midline sections were stained either with haematoxylin and eosin (H&E) or Toluidine Blue. Samples were coded and scored blind by Dr. Christopher Clarke (GlaxoSmithKline, Ware).

Knee joints from both treatment groups showed evidence of significant amounts of inflammation and joint destruction associated with the chronic inflammation (Figure 6.27). In all cases the pathological changes were unilateral, restricted to the left knee joint and present to a greater or lesser degree in each animal. There was marked periarticular inflammation involving the tendons and muscles present in all FCA-treated animals, and other changes observed included

mild synovial hypertrophy, fibrosis and pannus formation. These effects were consistent both within each group and between the two treatments. There did not appear to be any significant differences in the prednisolone treated animals. Scores for the histological criteria, shown in Table 6.1, were pooled for each animal and the median score taken for each group (Figure 6.28). There was no significant difference in the inflammation and joint destruction between the prednisolone (median score: 10) and vehicle (median score: 7) treated animals ($P = 0.13$; Mann-Whitney).

EFFECT OF NSAID INDOMETHACIN ON ESTABLISHED FCA-INDUCED UNILATERAL ARTHRITIS IN C57BL6 MICE

For additional validation of the model of murine FCA-induced unilateral arthritis a second “gold standard” for inflammation and pain relief was used. The non-steroidal anti-inflammatory, indomethacin, was used on mice with a chronically inflamed joint (Kelley *et al.*, 1997). Unilateral arthritis was induced in the left knee joint of C57BL6 mice and drug treatment carried out once the chronic inflammation had been established, seven days after the final injection of FCA (day 28).

The experiment was carried out in two groups of 8 week-old, male C57BL6 mice ($n=8$ per group). Animals were monitored both subjectively and objectively throughout the experiment to determine the extent of the inflammation and hyperalgesia/allodynia produced. Parameters were measured twice per week, once just prior to each injection of FCA and once midway between each injection. Once the unilateral arthritis was established in both groups the mice continued to be measured as above while being treated with either indomethacin (1 mg kg^{-1} subcutaneous daily) or vehicle. Drugs were coded and administered blind.

Knee joint diameter

The diameter of both left (injected) and right (uninjected) knee joints was measured throughout the course of the experiment. Chronic unilateral arthritis was induced in both groups and treatment with indomethacin or vehicle began on day 28, seven days after the final injection of FCA (Figure 6.29). Prior to the first FCA injection (day 0) there were significant differences in the right knee joint diameters ($P = 0.03$, unpaired t-test) but not the left knee diameters ($P = 0.09$; unpaired t-test) between the two groups.

Pre-treatment: In the chronically inflamed joints, 7 days after the final FCA injection (day 28) there was a significant increase in the left knee joint that was not seen in the contralateral limb in both groups ($19.4 \pm 3.1\%$ and $21.2 \pm 2.0\%$). The extent of the inflammation induced was similar for both groups as there was no significant difference in the diameter of the injected joint between groups ($P = 0.64$; unpaired t-test) – see Figure 6.29A-B.

Response to treatment: After seven days of treatment, at day 35, the previously swollen left knee decreased towards the diameter of the uninjected contralateral limb. Using the contralateral limb as an internal control, the chronic inflammation remained elevated in vehicle controls with the mean left knee diameter $19.3 \pm 2.0\%$ larger with respect to the right. Treatment with indomethacin decreased the difference between limbs to $8.3 \pm 2.9\%$, considered significant ($P = 0.007$; unpaired t-test; Figure 6.29C). However, examining each animal at day 35 for the change in the left knee diameter from day 0 showed that on average indomethacin treated animals ($+0.34 \pm 0.17$ mm) were not statistically different ($P = 0.15$; unpaired

t-test) from vehicle treated animals with a change from the starting diameter of $+0.64 \pm 0.10$ mm (Figure 6.29D).

Body weight

Prior to the induction of unilateral arthritis with FCA there was no significant difference in the starting body weights prior to the induction of unilateral arthritis with FCA ($P = 0.39$; unpaired t-test).

Pre-treatment: Once the chronic inflammation had been established at day 28 there was no difference in the average body weights between the two groups ($P = 0.81$; unpaired t-test; Figure 6.30). Furthermore, on average there was no significant difference between the two groups in the weight gained by each animal up to day 28 (3.3 ± 0.4 g and 3.8 ± 0.5 g; $P = 0.50$; unpaired t-test).

Response to treatment: Seven days of treatment with indomethacin did not alter the mean body weights, there was no significant difference at day 35 from vehicle controls for the average body weight ($P = 0.62$; unpaired t-test; Figure 6.30A). In addition, when comparing the change in body weight from day 0 for both indomethacin and vehicle treated animals; there was no significant difference in the weight gained (3.6 ± 0.6 g and 2.3 ± 0.7 g respectively; $P = 0.50$; unpaired t-test; Figure 6.30B).

Hyperalgesia/allodynia

Discomfort from walking during chronic inflammation

Pre-treatment: Although on day 28 all animals displayed chronic inflammation, there was no real sign of discomfort or impairment in walking for

either group. Each group contained some animals with very mild limping (3/8 mice for both groups; median score: 0; Figure 6.31A).

Response to treatment: After seven days of treatment with indomethacin there was no change in the number of animals showing limping behaviour (3/8 mice; 37%; median score: 0), which was not statistically different from the vehicle treated group ($P = 0.39$; Mann-Whitney; Figure 6.31B).

Response to noxious pressure on the knee joint

Pre-treatment: During chronic inflammation, at day 28, both groups were sensitised to a painful noxious stimulus. The sensitisation was restricted to the arthritic left knee joint with 8/8 animals (100%) in each group showing hyperalgesia (median scores: 1.5 and 2). There was no sensitisation in the right knee of any animal in either group (0/8; 0%). Between groups, the difference in the median hyperalgesia response in the left knee was not significant ($P = 0.40$; Mann-Whitney; Figure 6.32A) as both groups displayed similar levels of hyperalgesia.

Response to treatment: After 7 days of treatment with indomethacin, at day 35, there was a significant decrease in the hyperalgesia score for the left limb over that observed at day 28 (median: 0; $P = 0.007$, Mann-Whitney) – see Figure 6.32B. Indeed there was no significant difference in the hyperalgesia score between the left and right knee joints in indomethacin treated animals ($P = 0.40$; Mann-Whitney). Vehicle treated animals still displayed hyperalgesia in the chronically inflamed left knee joint when compared to the contralateral limb ($P = 0.007$, Mann-Whitney).

Histopathology

Knee joints were collected from recently killed animals from all groups on day 35 following 7 days of treatment with either indomethacin (1 mg kg^{-1}) or vehicle. The joints were processed as stated in the methods and midline sections were stained either with haematoxylin and eosin (H&E) or Toluidine Blue. Samples were coded and scored blind by Dr. Christopher Clarke (GlaxoSmithKline, Ware).

Examination of knee joints from both treatment groups showed the presence of significant amounts of inflammation, both within the joint and in the surrounding soft tissues (Figure 6.33). In all cases the pathological changes were unilateral, restricted to the left knee joint and present to a greater or lesser degree in each animal. There was marked periarticular inflammation involving the tendons and muscles present in all FCA-treated animals, and other changes observed included mild synovial hypertrophy, fibrosis and pannus formation. These effects were consistent both within each group and between the two treatments. There did not appear to be any significant differences in the indomethacin treated animals.

Histological scores were pooled for each animal and the median score taken for each group (Figure 6.34). There was no significant difference in the inflammation and joint destruction between the indomethacin (median score: 12) and vehicle (median score: 13) treated animals ($P = 0.28$; Mann-Whitney).

INDUCTION OF FCA UNILATERAL ARTHRITIS IN P2X₇ KNOCK OUT MICE

Background

Experiments were performed on 12 transgenic animals, which contained a mutation that disrupted the gene encoding the purinoceptor P2X₇, a receptor expressed primarily on immune cells and considered to play a role in chronic inflammation (North & Surprenant, 2000). Mice carrying a targeted null mutation of the P2X₇ gene were produced according to the published protocol (Conquet, 1995). Briefly, the P2X₇ gene was isolated from a genomic library obtained from 129/Sv mice, and sequencing of 5' exons permitted ligation of 2 fragments into the neomycin resistant "knockout vector", pGN (Le Mouellic *et al.*, 1990). Heterologous recombination of the resulting plasmid DNA into embryonic stem cells resulted in a disrupted P2X₇ gene. Disruption of the gene was confirmed by PCR and Western blot analysis in the resulting C57Bl6/129 hybrid mice. Six rounds of backcrossing onto a C57BL6 strain were performed resulting in 98.4% C57BL6 genetic background. These animals were supplied from GlaxoSmithKline (Stevenage, UK) to the University of Edinburgh under the appropriate Home Office licence. In addition to the transgenic animals (KO), this study included 12 wild type littermates (WT) to act as normal controls. All animals were transported to the University of Edinburgh from GlaxoSmithKline, (Stevenage, UK) when 8 – 10 weeks old and were housed for 1 week after transfer before any procedures were performed.

Knee joint diameter

Throughout the course of the experiment the animals were examined for evidence of swelling in the FCA injected knee joint, the contralateral uninjected knee joint, and the ipsilateral ankle joint – the latter to monitor for spreading of the inflammation to nearby joints. In addition to the regular subjective examination of the joints, both knees were quantitatively measured once per week prior to each injection of either FCA or HLP (Figure 6.35). Prior to any treatment (day 0) there was no significant difference between any of the groups in the diameter of either the left or right knee joints ($P = 0.74$ and $P = 0.84$ respectively; one-way ANOVA). To eliminate the contribution of the acute inflammation caused by each intra-articular injection of FCA or HLP, the analysis of the effect of the unilateral arthritis was examined at day 28 – seven days after the final injection of FCA, to restrict the study to measurement of chronic inflammation. Subjective examination of the mice that received repeated intra-articular injections of FCA into the left knee joint showed that there was substantial swelling in both KO and WT animals. Swelling was not seen in the joints of HLP-treated animals, in either in KO or WT groups. Quantitatively there was a significant increase in knee diameter of the FCA-injected joints in comparison with those that received HLP, both in the KO and WT animals ($P = 0.004$ and $P = 0.004$ respectively; Mann-Whitney). However, when comparing the KO and WT groups there was no significant difference between the two groups 7 days after the final injection of FCA ($P = 0.82$; unpaired t-test) (Figure 6.35A). This increase in joint diameter was not seen in the contralateral (right) knee joint (Figure 6.35B). Using the within animal control of the injected (left) and uninjected (right) knee joint it was possible to normalise the increase in the diameter of the injected

joint in comparison to the uninjected joint (Figure 6.35C). Comparing left and right joints showed that there was a significant increase in the diameter of the injected joint (left) of both KO and WT receiving FCA ($P = 0.0001$ for both; paired t-test) with an average increase of $24.1 \pm 1.6\%$ and $27.1 \pm 3.3\%$ respectively. These increases in joint diameter were not significantly different when comparing between the KO and WT groups ($P = 0.42$; unpaired t-test). The diameter of the injected joint at day 28 was compared with the diameter at day 0 in each mouse (Figure 6.35D) and the results showed that the injected knee joint of both KO and WT animals treated with FCA were larger than the starting diameter ($+1.12 \pm 0.09$ mm and $+1.29 \pm 0.17$ mm respectively) when compared to HLP injected controls (KO: $+0.04 \pm 0.05$ mm, WT: $+0.03 \pm 0.11$ mm). In both KO and WT the increase in joint diameter was significantly greater than that in the corresponding HLP controls ($P = 0.004$ and $P = 0.01$ respectively; Mann-Whitney). There was however no significant difference between KO and WT groups in the FCA treated group ($P = 0.41$; unpaired t-test).

Body weight

Throughout the course of the experiment body weight was measured as an indicator of normal eating, weight gain and overall health (Figure 6.36). All animals used were male, adults (8-12 weeks), but because animals came from different litters, the different breeding times meant that there was a wider range of starting weights than normally seen in stock animals. Although overall there was no significant difference in the mean weights at the start of the study prior to any treatment ($P = 0.07$; Kruskal-Wallis), the WT mice that were to be treated with FCA weighed significantly less when compared with KO mice that were to receive FCA ($P = 0.02$, Mann-Whitney), but not to the other two groups that were to be given HLP ($P = 0.09$

and $P = 0.21$ for KO and WT respectively; Mann-Whitney; Figure 6.36A). As the mean weight of the four groups, the measurement of weight gain in these animals was expressed as the increase in weight (weight at day 28 – day 0) for each individual mouse (Figure 6.36B). At day 28 groups treated with FCA gained less weight (KO: -0.25 ± 0.60 g, WT: 0.14 ± 0.35 g) when compared to HLP treated controls (KO: 2.83 ± 0.60 g, WT: 2.40 ± 0.93 g). The lack of weight gain in FCA treated animals was statistically significant in both KO and WT animals ($P = 0.004$ and $P = 0.02$ respectively; Mann-Whitney). However, there was no significant difference in weight gain in FCA treated groups when comparing KO and WT animals ($P = 0.59$; unpaired t-test).

Hyperalgesia/allodynia

Each intra-articular injection caused an acute hyperalgesia/allodynia response lasting for a couple of days and was present in both FCA and HLP injected mice. Discomfort in walking was manifest in a limping behaviour in associated with the injected limb that in some animals reached its maximum with a three-legged gait.

Discomfort in walking during chronic inflammation

Seven days after the final injection, when the acute effect of the injection had subsided there was a increase in the apparent discomfort associated with walking (Figure 6.37) in the majority of animals (7/8 (87%) for both KO and WT) that received repeated injections of FCA (KO median score: 2; WT median score: 2). This was not seen in any animals (0/8 (0%) both KO and WT) that were injected with HLP (KO median score: 0; WT median score: 0). This increase in apparent discomfort associated with walking was significant when comparing FCA injected

groups with HLP controls for both KO and WT animals ($P = 0.03$ and $P = 0.03$ respectively; Mann-Whitney) but there was no significant difference between KO and WT animals in FCA treated animals ($P = 0.59$; unpaired t-test).

Response to noxious pressure on the knee joint

Seven days after the final injection of FCA there was a significant sensitisation to a noxious pressure on the knee joint in animals that received FCA when compared to HLP controls in both KO and WT groups ($P = 0.004$ and $P = 0.01$ respectively; Mann-Whitney; Figure 6.38). The level of sensitisation was not significantly different in KO and WT FCA-treated animals ($P > 0.99$; unpaired t-test). The apparent hyperalgesia was almost exclusively observed in FCA-treated animals (8/8 (100%) animals for both groups; KO and WT median score: 2.5) with only one animal showing mild hyperalgesia in the HLP-treated animals (1/8; 12%). The hyperalgesia was restricted to the FCA-injected joint and was not seen in the contralateral limb.

Histopathology

Knee joints were collected from recently killed mice from all groups at day 28, seven days after the final injection of either FCA or HLP. The joints were processed as described in the methods (see section 6.2) and midline sections were stained either with H/E or toluidine blue. Mice treated with FCA showed a significant amount of inflammation and joint destruction characteristic of an arthritic condition. In KO or WT animals treated with FCA the most striking feature was the large amount of periarticular inflammation. This inflammation was present in the majority of tissue surrounding the joint including the ligaments, tendons and muscle.

As seen above in previous experiments with FCA-induced unilateral arthritis there was a moderate amount of inflammatory cell infiltration into the joint space and areas previously containing adipose tissue. Furthermore the large amount of cell debris in the joint space indicated a persistent and chronic inflammation of the joint. In both KO and WT animals there was also a moderate amount of pannus formation overgrowing the cartilage and causing cartilage destruction. Other changes included a noticeable amount of vascularisation in the tissue surrounding the joint. These changes were only present in the injected joint of FCA treated animals. HLP treated animals showed little or no joint destruction. The contralateral knee joints of both mice treated with either FCA or HLP showed no histological changes and presented as normal (untreated) healthy knee joints.

The histological score was pooled for each animal and the median score taken for each group (Figure 6.39). There was an increase in the histological score in FCA treated mice (KO median: 20.5; WT median 20.5) when compared to their HLP controls (KO median: 2.5; WT median: 3.5) that was significant for both KO and WT animals ($P = 0.004$ and $P = 0.008$ respectively; Mann-Whitney). There was no significant difference in the median histological score between KO and WT mice ($P = 0.59$; Mann-Whitney).

RECORDING FROM THE SAPHENOUS NERVE IN NORMAL AND ARTHRITIC KNEE JOINTS OF C57BL6 MICE

Neural recordings were carried out according to the final adopted methods detailed above in Section 6.2. A total of 10 normal and 6 arthritic C57BL6 mice (average weight: 28.0 ± 0.9 g; range: 24.5-31.9 g) were used for electrophysiological studies. Neural experiments were conducted on arthritic animals 7-14 days after the

final intra-articular injection of FCA and the mice showed an average knee diameter increase in the injected joint of 24.6 ± 3.3 %. Animals were anaesthetised with urethane (1.4 g kg^{-1}) and the trachea and right femoral artery were cannulated. Neural activity was recorded using a bipolar electrode on the saphenous nerve of the left limb that had been cut both proximally and distally to isolated the receptive field to the knee joint. Data recorded was analysed off-line using Spike2 software.

This was a pilot study and the successes rate for viable neural recordings was only 56% with 9 neural recordings made from 16 C57BL6 mice. Successful recordings were made from 17 afferents from 4 normal knee joints and 10 afferents innervating 3 arthritic knee joints. Classification of the afferents by conduction velocities was not possible due to limitations of size preventing the placement of the stimulating electrode on to the joint. However, probing the knee joint capsule with a hand-held plastic probe could mechanically activate all fibres tested and all responded to capsaicin (33-980 pmol) and as such were assumed to be C-fibres polymodal nociceptors (Figure 6.40). To prevent desensitisation of the preparation and effort was made to minimise mechanical probing of the joint and a minimum of 20 minutes was left between close-arterial injections of drugs.

Basal discharge of nociceptors innervating normal and arthritic knee joints

The small number of experiments conducted in both normal and arthritic knee joints did not permit an extensive examination of basal firing however 3/4 (75%) normal animals compared to only 1/3 (33%) arthritic animals showed spontaneous afferent activity. The rate of discharge in normal knee joints was 0.45 ± 0.42 impulses s^{-1} when compared to the value of 0.1 impulses s^{-1} for the single arthritic knee joint that showed spontaneous discharge.

Dose-related increase in the action potential discharge evoked by capsaicin and bradykinin

Both capsaicin and bradykinin evoked a dose dependent response from afferents innervating normal knee joints. Individual dose response curves were generated for each experiment for both capsaicin (n=3) and bradykinin (n=1; Figure 6.41 and Figure 6.42 respectively).

Features of the capsaicin and bradykinin evoked responses

Afferent discharge was evoked by capsaicin (33-980 pmol) in all fibres innervating both normal (17/17; 100%) and arthritic (10/10; 100%) knee joints. Similarly, bradykinin (94-2830 pmol) evoked an increase in afferent discharge from 10/14 (71%) fibres in normal joints but only 1/7 (14%) fibres in arthritic joints. Typical responses to capsaicin and bradykinin are shown in Figure 6.43. For both capsaicin and bradykinin the elements of the response, including delay to onset, duration, and the magnitude of the response were examined in more detail (Figure 6.44)

Capsaicin (330 pmol; n = 4) evoked a response with both a short delay to onset (3.6 ± 0.4 s) and duration (6.3 ± 3.2 s). The mean change in the discharge frequency generated capsaicin was 100.9 ± 38.5 impulses s^{-1} and the mean total afferent discharge for the response was 431 ± 132 impulses.

Bradykinin (940 pmol; n = 3) evoked a response that had a longer delay than capsaicin (6.3 ± 2.9 s) but had a similar average duration (6.9 ± 3.1 s). The mean change in the discharge frequency evoked by bradykinin was 28.7 ± 8.6 impulses s^{-1} and the mean total discharge was 244 ± 164 impulses.

6.4 DISCUSSION

The main finding in this component of the study is the result that it is possible to induce a chronic unilateral arthritis in both DBA/1 and C57BL6 mice using repeated intra-articular injections of FCA into the knee joint. The development of this murine model progressed from the rat FCA model of unilateral arthritis previously developed in this laboratory.

One of the aims of this project was to examine the induction of unilateral arthritis in particular transgenic mice, so the genetic background of the transgenic lines of interest influenced the choice of mouse strain. Furthermore we needed to establish a model that would produce a robust and consistent response across the strains tested. The P2X₇ knock out strain used in this study, and a strain with an alteration to a somatostatin receptor (not used in this study), were on DBA/1 and C57BL6 genetic backgrounds, respectively. These strains were also of interest because they have previously been used in the study of experimentally induced arthritis. DBA/1 mice spontaneously developing an RA like condition (Holmdahl *et al.*, 1992; Nordling *et al.*, 1992a; Nordling *et al.*, 1992b), as well as being the only strain that responds to collagen induced arthritis (Joosten *et al.*, 1999; Joosten *et al.*, 1996; Joosten *et al.*, 2000; Lubberts *et al.*, 2000; Wooley *et al.*, 1981); and C57BL6 mice have been primarily used in the development of antigen- induced arthritis (Brackertz *et al.*, 1977b; Brackertz *et al.*, 1977c; van de Putte *et al.*, 1983; van Lent *et al.*, 2000; van Meurs *et al.*, 1999).

The starting point of investigation was to study single sub-cutaneous injections of FCA around an ankle or knee joint in mice, as previously studied in the rat unilateral model (Birrell *et al.*, 1990a; Birrell *et al.*, 1990b; Birrell *et al.*, 1990c;

Donaldson *et al.*, 1993; Dowd *et al.*, 1997; Dowd *et al.*, 1998b). The mouse experiments produced an acute inflammatory response that resolved back to a baseline value within a matter of days. The arthritis was obtained in both the ankle and knee joints, but despite using increased concentrations of FCA, the inflammation following the single dose of FCA failed to become chronic.

Since it was not possible to directly transfer the single-dose FCA unilateral model from rat to mouse it was necessary to explore alternative methods for the induction of establishing unilateral arthritis in mice. These other methods we looked at included antigen induced arthritis (AIA) using mBSA (Klasen *et al.*, 1986; van den Berg *et al.*, 1982; van den Berg *et al.*, 1984), and repeated injections of FCA. Both models involved learning the technique of intra-articular injections into the mouse knee joint, which was undertaken, under the direction of Dr. Leo Joosten in the Department of Rheumatology, University of Nijmegen, The Netherlands.

Attempts to induce unilateral arthritis with AIA using mBSA injected into the left knee joint, following immunisation were undertaken in C57BL/6 and DBA/1 mice. As was the case with a single injection of FCA, it was not possible to produce a chronic inflammation of the knee with AIA. Mild inflammation, in the order of a 5-10% increase in the diameter of the injected joint, could be induced acutely in both strains, but the inflammation resolved back to baseline levels within a week for both strains.

Another model used by Dr. Joosten involved an intra-articular injection of purified bacterial cell walls from group-A *Streptococci* (SCW) (van den Broek *et al.*, 1988). A single intra-articular injection of SCW evoked an acute inflammation that peaked approximately 4 days after injection and persisted for up to a week (van den

Broek *et al.*, 1988). Furthermore, it was possible to induce a more chronic inflammation by repeated injections of the SCW (Dr. Joosten - personal communication). Our group has experience using FCA and is familiar with its use *in vivo*, so we speculated that it might prove possible to substitute the bacterial components of the *Mycobacterium tuberculosis* present in the FCA for the SCW, and use FCA to induce a chronic unilateral inflammation by repeated intra-articular injections of FCA into a knee joint.

As the results show, four injections of 5 μ l of FCA given at weekly intervals proved able to evoke chronic unilateral arthritis in both DBA/1 and in C57BL6 mice with similar progression of the condition in both strains. In the initial phase acute inflammation occurred after each injection; this flared and then resolved within seven days, as seen with the single sub-cutaneous injections. However, with each subsequent injection the acute phase response was greater and the inflammation resolved less and less such that one week after the final injection inflammation persisted in the injected joint and chronic inflammation was thus established. The inflammation was restricted to the injected joint and with the doses of FCA used there was no swelling in the contralateral leg, such as can be induced with a sufficiently high dose of FCA in the rat (Donaldson *et al.*, 1993). Further studies will be done to determine where it is possible to produce the crossover of inflammation in mice by increasing the dose of FCA.

The knee diameter was the primary measure used to assess the extent of the inflammation in the mice, however other measures, both quantitative and qualitative, were also used. For example, the course of inflammation was tracked by measuring the increase in temperature associated with increased blood flow in arthritic joints

(Abbot *et al.*, 1996; Bray *et al.*, 1996; Haywood & Walsh, 2001) using an infrared thermometer (Braun, ThermoScan). This was tried for a number of experiments (data not shown in this study) but it proved impossible to obtain consistent difference between normal and inflamed joints and there was a high variability between animals. The measurements were greatly affected by ambient temperature with large fluctuations from day to day that correlated with changes in room temperature. Normalising responses to a ratio of injected to uninjected joint still showed a large variability both between animals and within animals tested on consecutive days. Further work examining inflammation in terms of changes in local blood flow in the knee joint could be developed using laser Doppler perfusion imaging (Ferrell *et al.*, 1996) or ⁹⁹Technicium uptake (Kruijsen *et al.*, 1981).

In addition to the subjective measures of apparent motility allodynia and hyperalgesia, as measured by a reduced threshold for application of increasing moderate to intense pressure to a joint by squeeze, we briefly examined a number of alternative measures including the ability of the animals to grasp a bar and the ability to maintain position on a series of gradients and surfaces (Williams *et al.*, 1993). These did not provide useable information and were not pursued following pilot studies.

As stated above we examined the weight bearing allodynia subjectively by monitoring the gait of the animal and its willingness to apply weight to the injected limb. Following each intra-articular injection there was a period of days where the animals displayed a limping behaviour where, in extremis, an animal guarded one leg and only walked on three legs. The allodynia typically lasted less than a week, and animals walked normally by the next injection in the series. Limping following each

injection was present in both DBA/1 and C57BL6 irrespective of FCA or HLP treatment, and was likely an artefact of the injection procedure.

Both strains could bear weight on the injected limb in the chronically inflamed joint while walking. The examination of changes in gait might not provide as much information on the apparent analgesia as the mice can distribute their body weight across all four limbs. Measurements made in static animals could force the mice to distribute their weight between a normal and a chronically inflamed limb. As mentioned above, in the rat model of unilateral FCA arthritis weight bearing in a static animal can be assessed using a sensitive double balance (Clayton *et al.*, 1997). This method is capable of detecting slight changes in weight distribution (allodynia/hyperalgesia) that are not significant enough to evoke limping or guarding of the injected joint. In mice, it was not possible to examine weight bearing objectively, as in the rat, due to limitations of the equipment. The double balance was not sufficiently sensitive to allow the measurement of the weight bearing, and mice are very mobile and difficult to restrain, so we were not able to ascertain weight bearing in the static animal. One project for future research would be the development of a sensitive weight bearing apparatus for mice, addressing the problems of restraining the mice while maintaining access to the hind limbs for measurement.

Inflammation causes hyperalgesia and this was assessed in terms of the sensitisation of the knee joint to a noxious stimulus, in this case squeezing the joint between thumb and forefinger. Again due to the very small size of mouse joints it was not feasible in the time available to develop a quantitative measuring device for hyperalgesia as has been previously used in our laboratory in rats. Measurements

therefore were based on subjective scoring and conducted blind. As stated in the Methods, attempts to quantify the procedure were made by reproducing the squeeze pressure applied to the joint on a pressure transducer. The average result on the transducer divided by the average surface area of the knee joint provided an average force applied (in g/mm^2) for each score. The results show that in both DBA/1 and C57BL6 mice there is a sensitisation that is restricted to the injected limb, permitting an internal control for hyperalgesia. Future work is planned to provide a quantitative measure of the hyperalgesia in the joint using some form of pressure transducer linked to an inflatable cuff or sensitive strain gauge bonded onto callipers that can be automatically closed to apply gradually-increasing force to the joint.

The behavioural changes in the response to pain as well as the gross changes in the inflamed joint were correlated with the pathology associated with FCA treated joints. Overall, the extent of the inflammatory changes in both DBA/1 and C57BL6 mice was consistent with a mild to moderate arthritis that was restricted to the injected limb. Lack of any pathology in the contralateral limb or the ipsilateral ankle (data not shown here) meant that the inflammation was indeed isolated to the injected knee joint. The results show that the primary feature of the histology is the presence of a large amount of peri-articular inflammation – inflammation of the tissue surrounding the joint capsule and including the muscle, tendons and ligaments. The smaller amount of fibrosis present in peri-articular inflammation, in comparison with the inflammation within the joint, shows that the inflammation began within the joint and later moved to extra-articular tissues. The large amount of extra-articular inflammation and the fact that it was less well established than that seen within the joint was probably the result of later injections. Large amounts of swelling in the

joint, particularly prior to the third and fourth FCA injections, meant that it was not always possible to identify the patella tendon to ensure that the injection was made directly into the joint capsule, and it is possible that the later injections were made into the surrounding tissue where they would produce a large amount of extra-articular inflammation.

The arthritis present appeared to be more characteristic of an OA as opposed to RA (Rosenberg, 1999). Characteristics of a rheumatoid type arthritis include the presence of pannus and although in some cases there was a small amount present it was not a prominent feature as is seen in some other mouse models including collagen induced poly-arthritis (Stuart *et al.*, 1984; Trentham *et al.*, 1977)

In both DBA/1 and C57BL6 strains there was a significant correlation between the inflammation induced and the pressure required to get the animal to withdraw the limb. This correlation indicates that drugs that decrease the amount of inflammation will also have an effect on the hyperalgesia present in the joint. Proving the model with two “gold standards” further supported this. The “gold standards” used in our study were a steroid, prednisolone, and a non-steroidal anti-inflammatory (NSAID) indomethacin, both capable of reducing inflammation and pain in a number of animal models and in clinical situations (Kelley *et al.*, 1997). Ideally for the drugs to be considered true standards they should have been administered orally but a limitation in my Home Office Personal Licence prevented me from being able to administer the drugs in this way. Problems were experienced with determining the most effective route of administration particularly with the NSAID, indomethacin. Initially, it was thought that both prednisolone and indomethacin could be administered i.p. However the initial dose of indomethacin

(10 mg/kg) was highly toxic by this route as was a ten-fold reduction (1 mg/kg). It was decided that it should be given by the sub-cutaneous route, which proved to be effective. Both prednisolone and indomethacin at the doses given significantly reduced the inflammation in the injected limb and in the case of prednisolone the knee diameter was restored to pre-FCA injection values. Furthermore, both drugs were able to significantly reduce the hyperalgesia in the inflamed joint to the level of the contralateral limb or to HLP injected controls. Although, the drugs were able to reduce inflammation and pain the histology did not show any significant difference between drug treatment and the corresponding controls. This is consistent with the fact that the treatment was short-duration and started in an established arthritis where there is very little in terms of reversal of the pathology (Dr. Clarke – personal communication). Further work will be required to determine whether with longer-term steroid/NSAID administration it is possible to resolve the pathological changes. It would also be interesting to study whether the induction of arthritis can be prevented by administering these same drugs prior to, or in conjunction with, the FCA injections. The reduction of inflammation and hyperalgesia by the two drugs in the established arthritis means that the FCA-induced unilateral arthritis model could be useful for predicting effective drugs for treating arthritis and the development of drugs with less severe side effect profiles than that associated with chronic use of both steroids and NSAIDs.

Recent reports have shown that the pro-inflammatory cytokine IL-1 plays a definitive role in inflammatory hyperalgesia (Cunha *et al.*, 2000), and in conjunction with TNF α is responsible for the inflammation and joint destruction associated with RA (reviewed in Carteron, 2000; van den Berg, 2000a; van den Berg, 2001c).

Furthermore clinical trials have begun using anti-TNF α and anti-IL-1 as treatment for the condition (Feldmann & Maini, 2001; Maini & Taylor, 2000; van den Berg, 2001a) showing the importance of these cytokines in the human condition. For this reason future experiments should look at the role of TNF α and IL-1 in this model of arthritis to determine its relevance to human RA.

As stated above the purinoceptor P2X₇ receptor was hypothesised to have a role in the development of arthritis through the release of IL-1 from activated immune cells. The results in the unilateral FCA mouse model showed there was no significant difference between KO and WT mice in terms of the ability to induce a chronic arthritis or the pain associated with that arthritic condition. Furthermore, there was no difference in the histological score, meaning that the extent of the inflammation and joint destruction was similar in both. Therefore in this particular model there is no apparent link between the P2X₇ receptor and the ability to induce a chronic inflammation using FCA. Although ATP has been shown to be a potent trigger for the release of IL-1 from inflammatory cells (Ferrari *et al.*, 1997a; Grahames *et al.*, 1999) it is unknown what proportion of the total IL-1 released during inflammation is mediated by P2X₇ stimulation and it may be that a significant amount of IL-1 is still produced in the arthritic joint. In order to determine the level of IL-1 released as a result of P2X₇ activation it would be interesting to examine IL-1 levels in the synovium of both KO and WT animals in response to FCA arthritis. Also, as with most knockout mice it is possible that as the receptor was knocked out during development there was compensation from other systems allowing the inflammation to perpetuate – potentially through the release of IL-1, so although the evidence doesn't support the hypothesis, it is not definitive.

Recent reports have shown that the P2X₇ receptor is expressed more widely than previously reported by Collo *et al.* (1997) and is present in the normal adult in both the brain and spinal cord and appears to have functionality not restricted to the release of inflammatory or cytolytic mediators from macrophages (Deuchars *et al.*, 2001). The P2X₇ receptor has been located on the presynaptic terminals of excitatory neurons in the CNS and in motor nerve terminals in the PNS (Deuchars *et al.*, 2001). This new role of P2X₇ in the facilitation of excitatory synaptic transmission particularly in the spinal cord means that it can now be investigated both in the periphery for the initiation of an inflammatory response and centrally as to its role in the modulation of synaptic transmission under inflammatory conditions.

One of the other objectives of this project was to be able to develop a way to complement information from behavioural studies by obtaining direct evidence through recording from sensory afferents in the knee joint, much as is done in the rat model (Dowd *et al.*, 1998a; Dowd *et al.*, 1998b; Gauldie *et al.*, 2001). Much of the time spent on this particular component of the thesis involved developing a protocol that was able to maintain a mouse under adequate anaesthesia for a full day, devising methods for recording from joint afferents, and the ability to administer the drugs to a joint by close arterial injection. The Spike 2 software enabled the multi-fibre recording to be analysed by and separating out the individual spikes that make up the response - as was used in the recordings from joint afferents in the rat.

The results show that it is possible to record from both normal and arthritic knee joints in the mouse. Further characterisation is required to definitively identify the fibre types being recorded, however as stated previously, the ability to activate these fibres both mechanically and with algogens such as capsaicin and bradykinin as

well as the long spike duration leads one to assume that they are C-fibre polymodal nociceptors (Gee *et al.*, 1999; Iggo, 1978; Wall & Melzack, 1999).

It has previously been shown, in Section 3.3, that in the rat basal discharge is present in a higher proportion of arthritic joints when compared to normal joints. This is consistent with the accepted theory that spontaneous firing is present in a higher proportion of arthritic joints when compared to normal joints. Furthermore, in joints where there is spontaneous firing present the discharge frequency is higher in the arthritic joints. When looking at the basal discharged recorded from both normal joints in the mouse there appears to be a higher proportion of spontaneous afferents in normal joints when compared to arthritic joints. The small number of experiments in both normal and arthritic animals is likely the reason for the discrepancy and further experiments will be required to verify this finding.

For both capsaicin and bradykinin a true maximum was not established for the dose response curves because high doses of both compounds, particularly capsaicin, are capable of desensitising both the response (Szallasi & Blumberg, 1999).and in some cases the entire preparation (personal observation). For this reason, and the difficulty in setting up the preparation, it was decided to use doses that would evoke a response but not produce desensitisation. Therefore, ED₅₀ values were not calculated as they did not provide a meaningful evaluation of the response. Elements of the response to both capsaicin and bradykinin appear to be roughly similar to those seen in the rat. The short delay to onset and short duration are typical of a capsaicin response in the rat, as is the slightly longer delay to onset of the evoked response to bradykinin.

The results presented in this study are preliminary and many more experiments are required both to refine the technique and to develop a coherent picture both of the types of afferents involved and their pharmacological characteristics. However, what has been demonstrated is the ability to activate and record from the sensory afferents that innervate the mouse knee joint - in normal and arthritic animals. The fact that these fibres could be activated by capsaicin implies that they contain C-fibres with VR-1 receptor gated ion-channels and, because bradykinin was effective on the same afferents, GPCRs are also intimately associated with these polymodal nociceptors. Much more investigation is required to determine the pharmacological profile of these afferents, but the basis for doing so has been established.

Many of the problems encountered during studies on murine joint nociceptors are directly related to the size difference between rat and mouse. It was impossible during the course of experiments to measure conductance velocities in the fibres recorded from because there was insufficient room around the joint to be able to put the stimulating electrode on the receptive field. Furthermore, it was not feasible to monitor blood pressure during the experiments because of problems in obtaining adequate BP signals from the fine catheters (high resistance) that were used for cannulating arteries. There are a number of refinements that need to be made in order for the technique to yield valuable results, but the pilot work has been very encouraging..

In conclusion these studies show it is possible to induce a chronic, unilateral joint inflammation in both DBA/1 and C57BL6 mice by repeated intra-articular injections of FCA. The pathology present in these joints is consistent with a mild to

moderate arthritis and it responds well to both prednisolone and indomethacin. Furthermore, I have developed a technique for recording from branches of the saphenous nerve innervating the mouse knee joint and can elicit responses that have been recorded from sensory nociceptors that, using known algogens such as capsaicin, are similar to those obtained in rat. Neural recordings, in conjunction with the unilateral model of arthritis, will allow examination of the effects of drugs and genetic manipulations on behavioural measures of pain as well as their effects on nociceptive transmission in both normal and chronically inflamed joints. This should provide some insight into the relationship between inflammation and nociception in chronic inflammatory conditions such as arthritis.

TABLES AND FIGURES

Table 6.1 – Criteria for histopathology scoring

Criteria on which histological sections were examined blind to evaluate the extent of inflammation and joint destruction. Each variable was scored arbitrarily on an analog scale 0 (mild) to 5 (severe).

Synovium	<ul style="list-style-type: none"> • Synovial hypertrophy • Oedema • Vascularity
Synovial inflammation	<ul style="list-style-type: none"> • Mononuclear cells • Polymorphonuclear cells • Fibrin/debris • Fibroplasia/fibrosis
Cartilage	<ul style="list-style-type: none"> • Pannus • Erosion/destruction • Cartilaginous hyperplasia
Bone	<ul style="list-style-type: none"> • Resorption/destruction • New bone formation • Ankylosis
	<ul style="list-style-type: none"> • Periarticular inflammation

Table 6.2 - Explanation of hyperalgesia scores resulting from a joint squeeze

Applying the squeeze between thumb and forefinger to a pressure transducer and taking the average measurement for each hyperalgesia score it was possible to estimate the pressure applied to the joint. The average pressure applied was divided by the average knee joint area to get an approximate force applied in g/mm^2 .

Hyperalgesia Score	Description	<u>Approximate Force</u> (g/mm^2) n = 12
0	Severe squeeze	> 60
1	Moderate squeeze	48.0 ± 0.8
2	Mild squeeze	35.5 ± 1.2
3	Touching or light squeeze	24.5 ± 1.2

Figure 6.1 – Schematic of the experimental set up for recording from saphenous nerve in mice

Schematic of the experimental set up for recording neural activity from sensory afferents in the saphenous nerve that innervate the mouse knee joint. Cannulas were inserted in the trachea and the right femoral artery for the close-arterial injection of drugs. The left limb was fixed to a solid support and the skin was tied to a small brass ring to form a cavity, which is filled with heavy liquid paraffin, producing an electrically isolated system. The saphenous nerve was cut above and below the entry point of the MAN and neural activity was recorded in the same way as for the rat – see section 2.2.

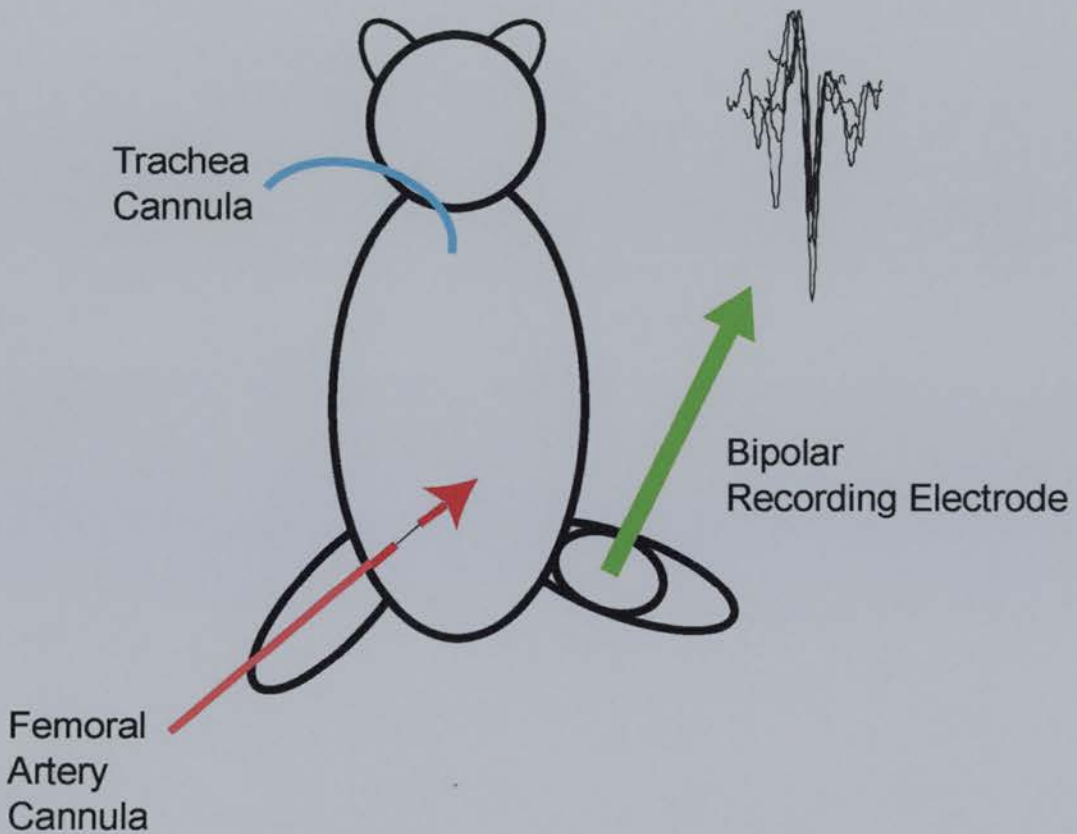


Figure 6.2 – Effect on joint diameter of a single sub-cutaneous injection of FCA over the ankle of DBA1 mice.

Diameter of the A) ipsilateral (left; injected) and B) contralateral (right; uninjected) ankle joints of animals receiving (at arrows) no injections (control, \triangle), or single sub-cutaneous injection of either FCA (\bullet ; 50 μl of a 1 mg ml^{-1} suspension) or HLP (\circ ; 50 μl). C) Increase in the left knee diameter expressed as a percentage of the right knee diameter, pooled data from comparisons of individual animals. Each data point represents the mean \pm SEM.

A single injection of FCA produced an acute inflammation but was unable to evoke a chronic arthritis.

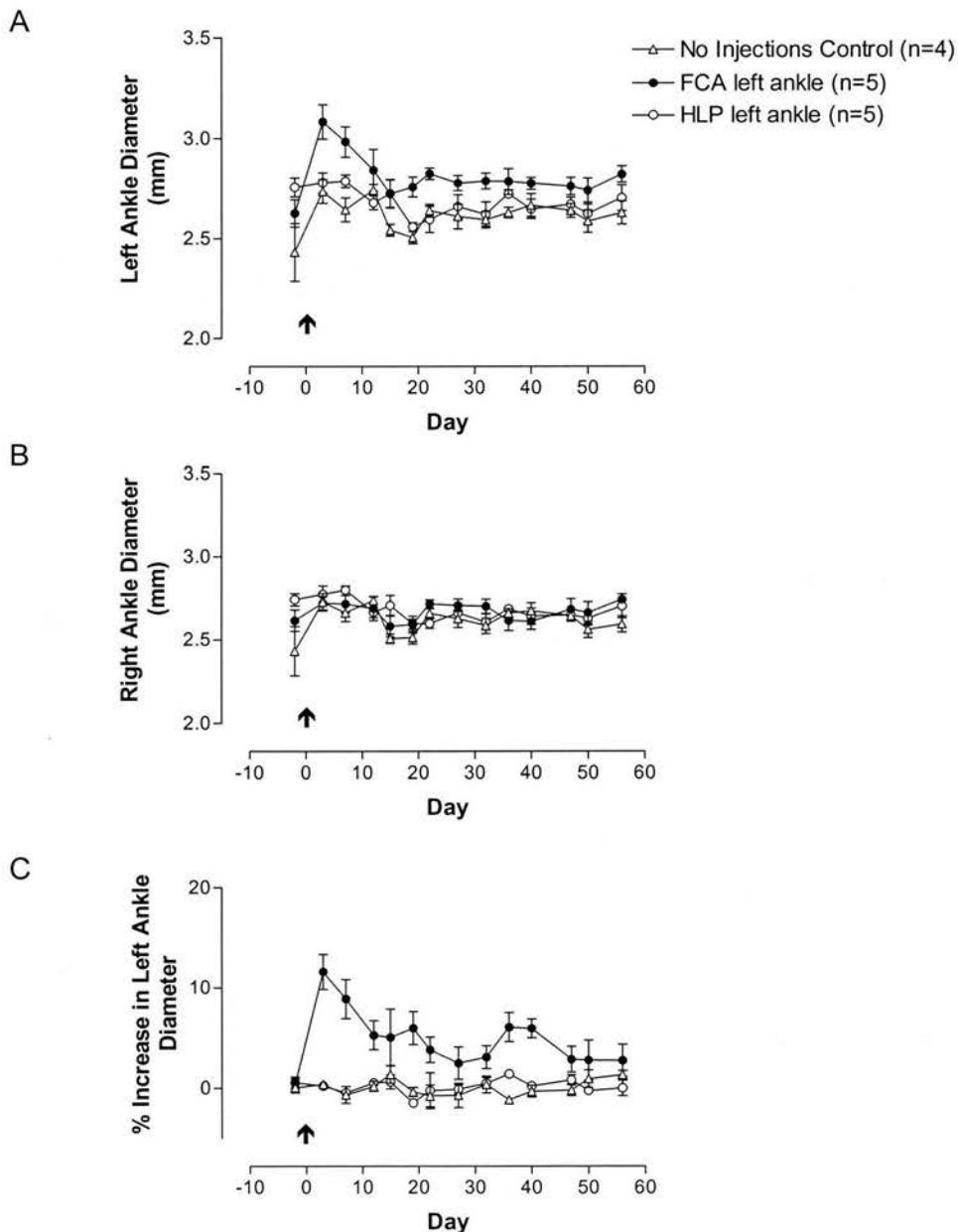


Figure 6.3 – Effect on joint diameter of a single sub-cutaneous injection of FCA (or a single plus a booster injection) over the knee of DBA/1 mice.

Diameter of the A) ipsilateral (left; injected) and B) contralateral (right; uninjected) knee joints of DBA/1 mice receiving (at filled arrows) a single sub-cutaneous injection over the knee of HLP (\circ ; $50\ \mu\text{l}$), FCA at a dose of either $100\ \mu\text{g}$ (\bullet ; $50\ \mu\text{l}$ of $2\ \text{mg ml}^{-1}$ suspension) or $150\ \mu\text{g}$ (\blacktriangle ; $50\ \mu\text{l}$ of $3\ \text{mg ml}^{-1}$ suspension) or FCA (\blacksquare ; $50\ \mu\text{l}$ of $3\ \text{mg ml}^{-1}$ suspension) plus a booster injection of FCA at day 21 (empty arrows; $50\ \mu\text{l}$ of $2\ \text{mg ml}^{-1}$ suspension). C) Increase in the left knee diameter as a percentage increase over the right knee diameter, pooled data from comparisons of individual animals. Each data point represents the mean \pm SEM.

A single FCA injection was incapable of inducing a chronic inflammation even at a dose of $150\ \mu\text{g}$. Furthermore a booster injection of FCA at day 21 does not produce a chronic arthritis.

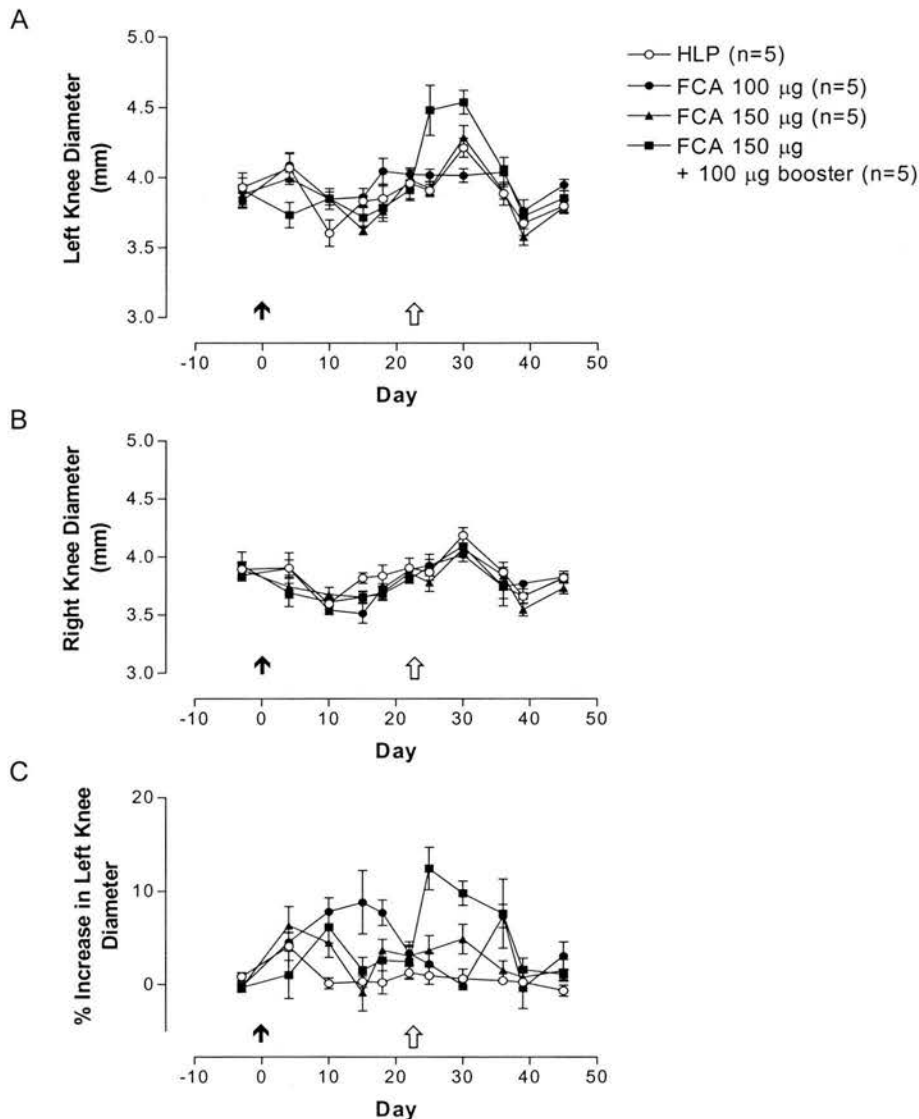


Figure 6.4 – Effect on knee diameter of mBSA-induced inflammation in the knee of C57BL6 mice.

Diameter of the A) ipsilateral (left; injected) and B) contralateral (right; uninjected) knee joints of mice receiving an intra-articular challenge injection of mBSA (●; 6 μ l of a 10 mg ml⁻¹ solution) or saline control (○; 6 μ l) 21 days following immunisation with mBSA. C) Increase in the left knee diameter as a percentage increase over the right knee diameter, pooled data from comparisons of individual animals. Each data point represents the mean \pm SEM. C

Chronic arthritis could not be induced with a challenge of mBSA by i.art injection.

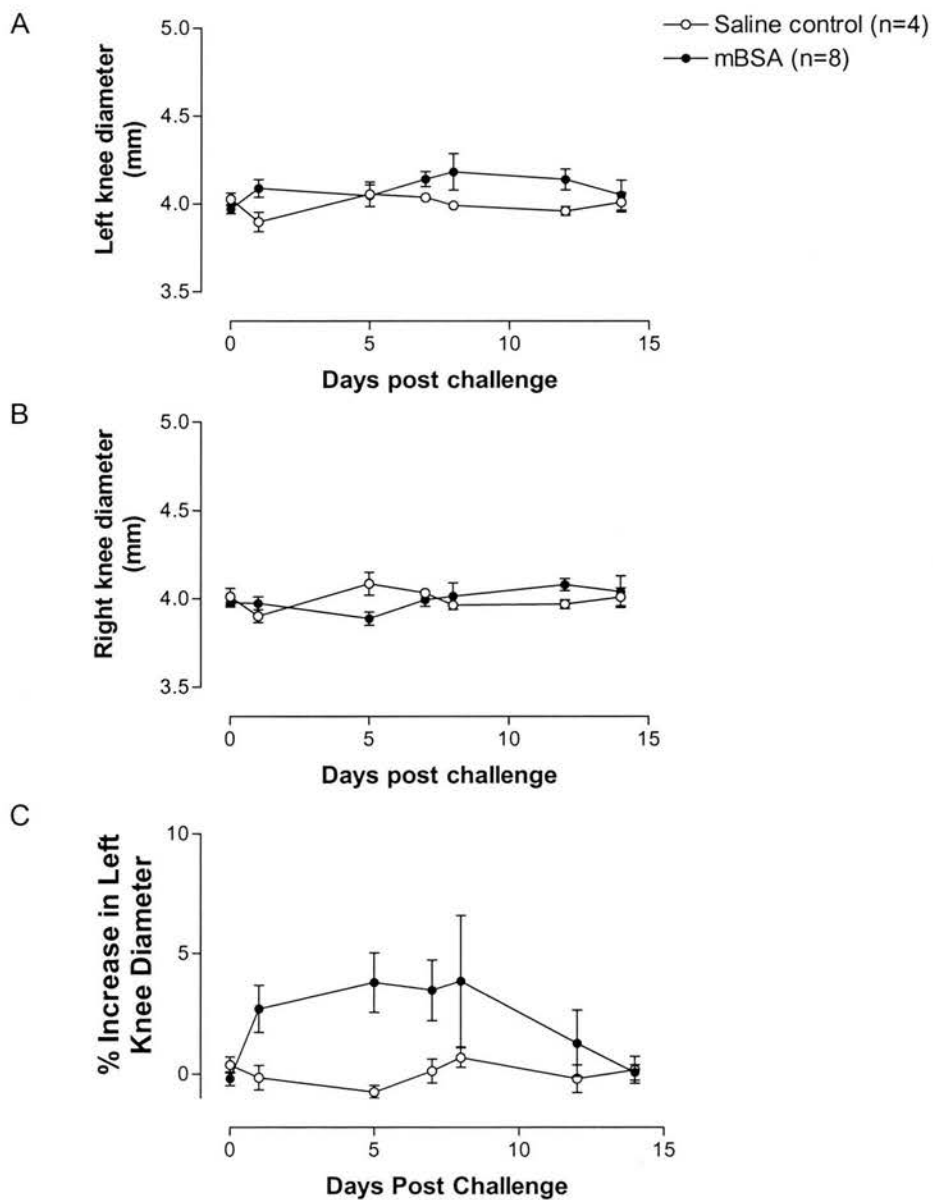


Figure 6.5 – Effect on joint diameter of mBSA-induced inflammation in the knee of DBA/1 mice.

Diameter of the A) ipsilateral (left; injected) and B) contralateral (right; uninjected) knee joints of mice receiving an intra-articular challenge injection of mBSA (●; 6 μ l of a 10 mg ml⁻¹ solution) or saline control (○; 6 μ l) 21 days following immunisation with mBSA. C) Increase in the left knee diameter as a percentage increase over the right knee diameter, pooled data from comparisons of individual animals. Each data point represents the mean \pm SEM.

Inflammation induced by an i.art. challenge using mBSA did not become chronic in nature.

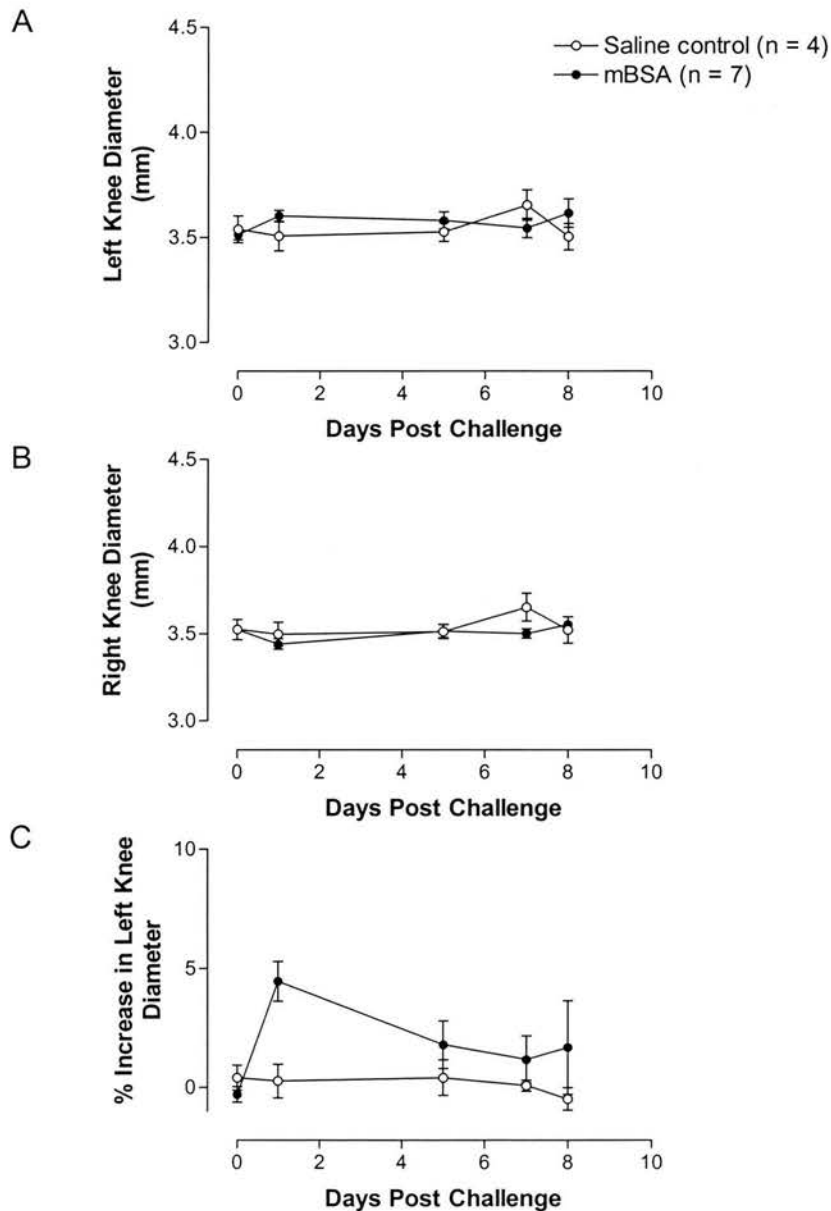


Figure 6.6 – Effect on joint diameter of repeated i.art. injections of FCA into the knee of DBA/1 mice.

Diameter of A) ipsilateral (left; injected) and B) contralateral (right; uninjected) knee joints of mice receiving repeated intra-articular injections (at arrows; 1 per week for 4 weeks) of FCA (●; 5 μ l of 1 mg ml⁻¹ suspension) or HLP (○; 5 μ l). C) Increase in the left knee diameter as a percentage increase over the right knee diameter, pooled data from comparisons of individual animals. D) Change in the left knee diameter of each animal as compared to the starting value (day 0). Each data point represents the mean \pm SEM.

A chronic inflammation was induced in DBA/1 mice by repeated i.art. injections of FCA.

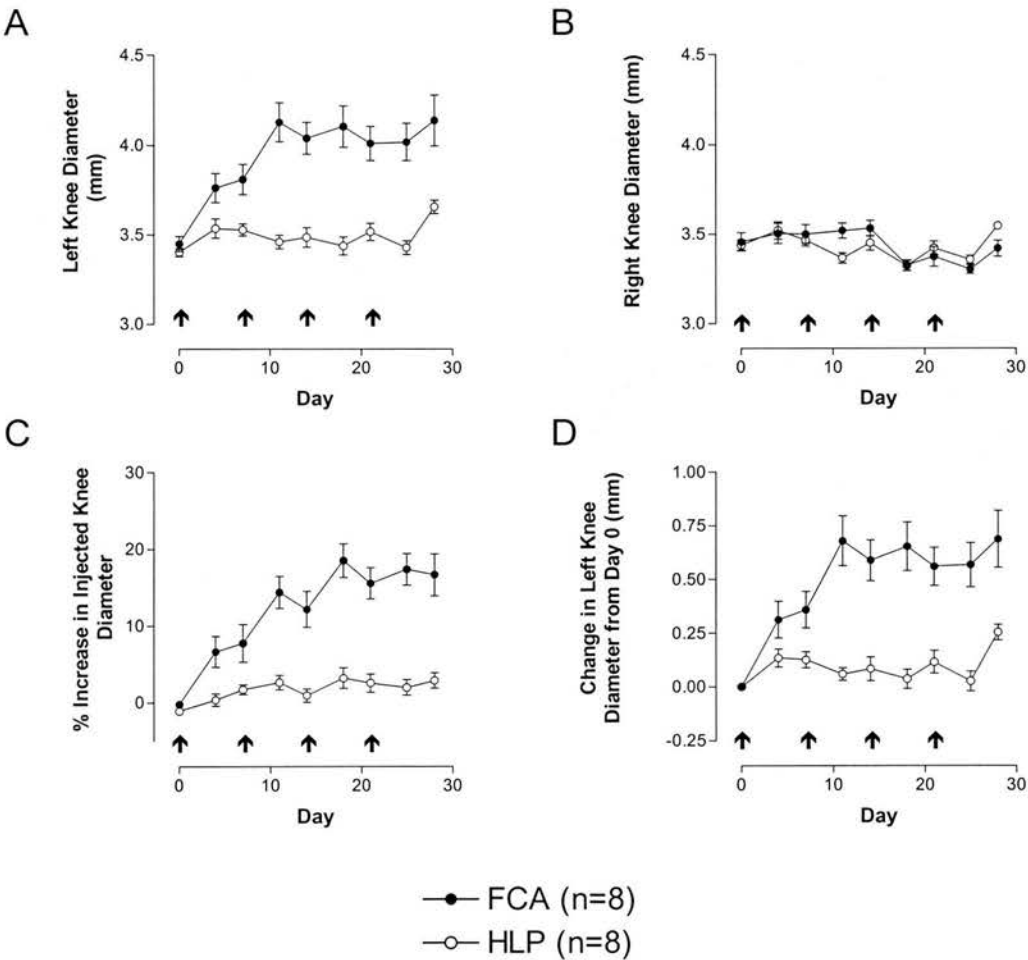


Figure 6.7 – Schematic of inflammatory response to repeated intra-articular injections of FCA

There are two components to the inflammatory response induced by FCA. The first component is an acute phase that immediately follows each injection (at arrows) and results in a large increase in swelling in the knee joint. The second phase is a more chronic phase that exists once the acute phase has resolved. With each injection the swelling produced in the acute phase is significantly greater. Furthermore, after the second a subsequent injections the acute inflammation resolves but never to the starting diameter indicating additional changes. After 4 injections the joint size has increased dramatically and remains swollen as the accumulated changes have induced a chronic inflammation.

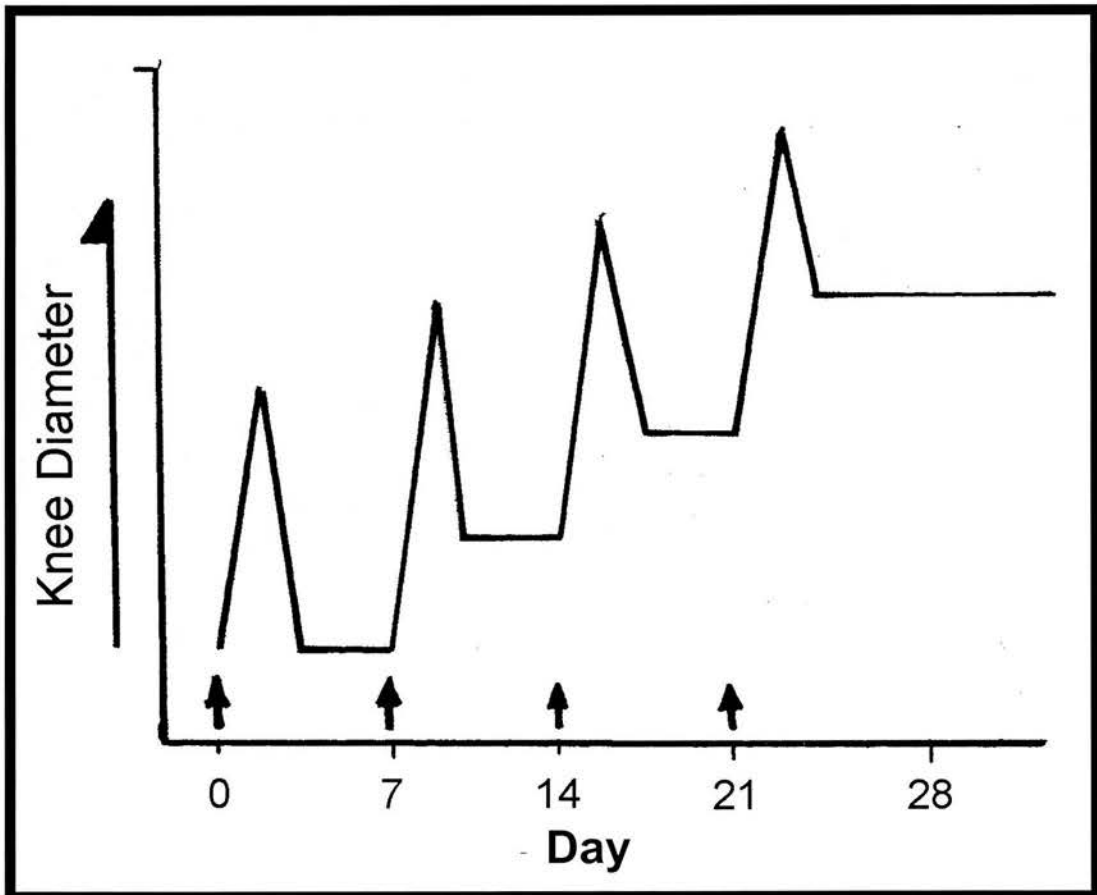


Figure 6.8 – Effect of repeated injections of FCA on body weight in DBA/1 mice

A) Body weight and B) change in body weight from day 0, in DBA/1 mice receiving repeated intra-articular injections (at arrows; 1 per week for 4 weeks) of FCA (●; 5 μ l of 1 mg ml⁻¹ suspension) or HLP (○; 5 μ l). Each data point represents the mean \pm SEM.

Treatment with FCA did not significantly alter the ability of DBA/1 mice to gain weight.

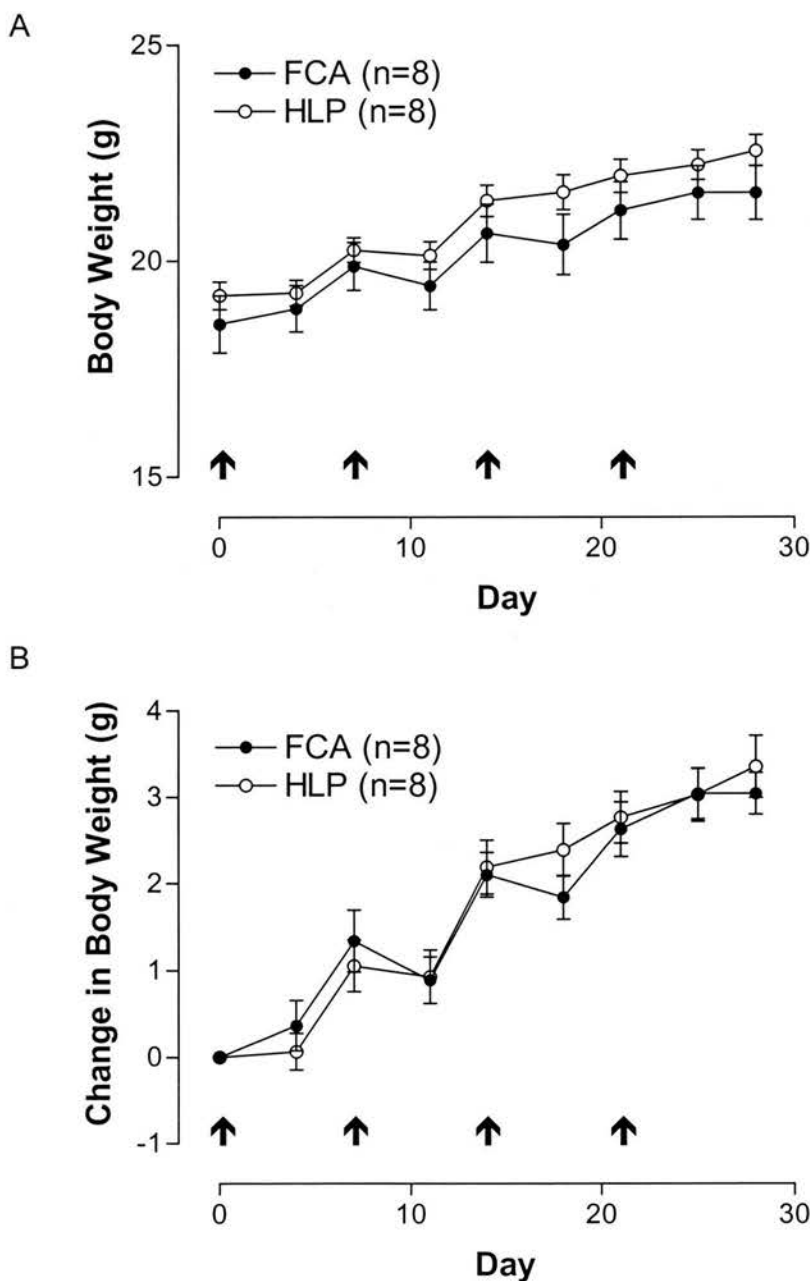


Figure 6.9 – Effect of repeated injections of FCA on mobility in DBA/1 mice

Score of individual DBA/1 mice and the median (thick line) of the discomfort associated with walking 7 days after receiving repeated intra-articular injections (1 per week for 4 weeks) of FCA (5 μ l of a 1 mg ml⁻¹) or HLP (5 μ l). The mice were subjectively scored on a 0-3 scale (3 maximum) for signs that they were in pain when bearing weight on the injected limb.

There was no significant difference between FCA and HLP treated groups in the ability to walk normally ($P = 0.62$; Mann-Whitney).

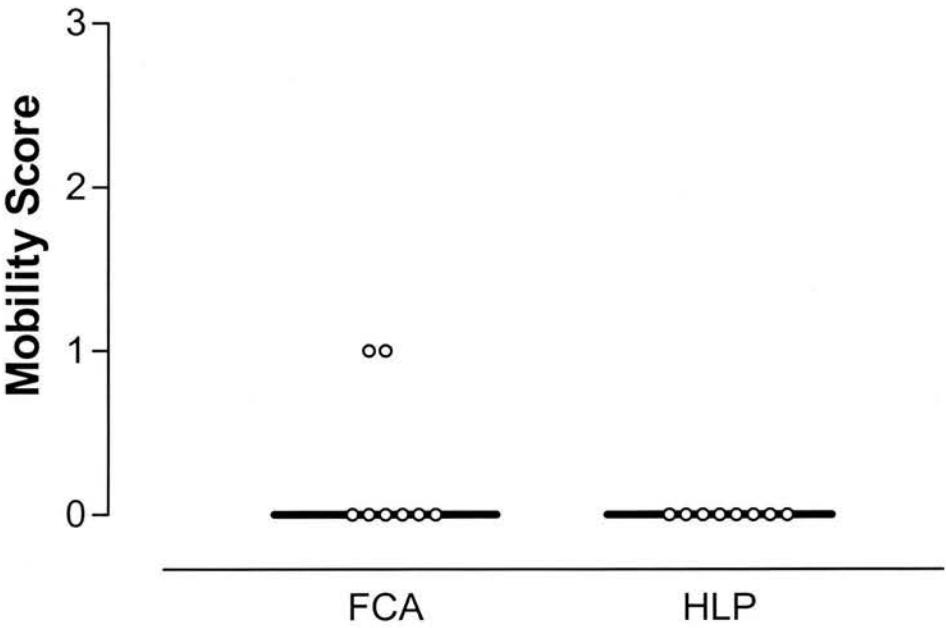
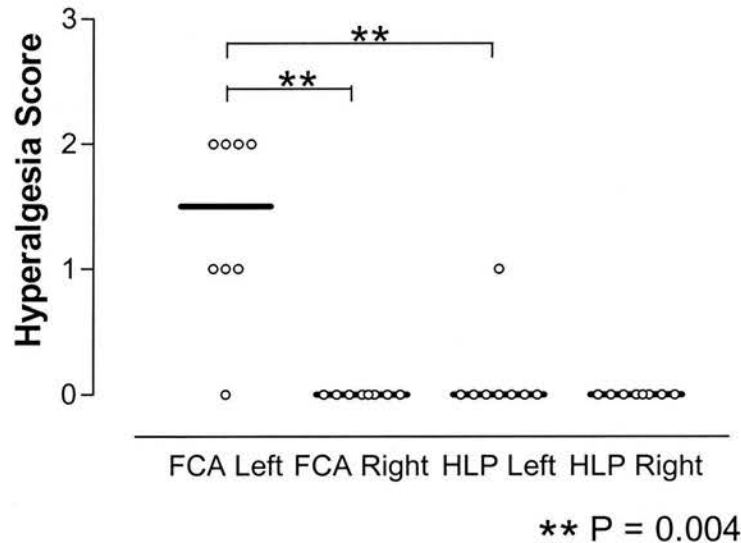


Figure 6.10 – Effect of FCA on hyperalgesia evoked by noxious pressure applied to the knee joint in DBA/1 mice

A) Individual hyperalgesia scores and median (thick line) in response to pressure applied to the ipsilateral (left; injected) or contralateral (right; uninjected) knee joint in DBA/1 mice 7 days after repeated injections (1 per week for 4 weeks) of FCA (5 μ l of a 1 mg ml⁻¹) or HLP (5 μ l). Joints were subjectively scored on a 0-3 scale (3 maximum) for withdrawal from the noxious stimulus. B) Correlation of the hyperalgesia score and the left knee diameter as a percentage increase over the right knee diameter, pooled data from comparisons of individual animals.

There was a significant increase in the median hyperalgesia score for animals that were treated with FCA (** $P = 0.004$, Mann-Whitney). Furthermore there was a significant correlation between the knee joint inflammation and the hyperalgesia score ($P = 0.0003$, Spearman rank correlation).

A



B

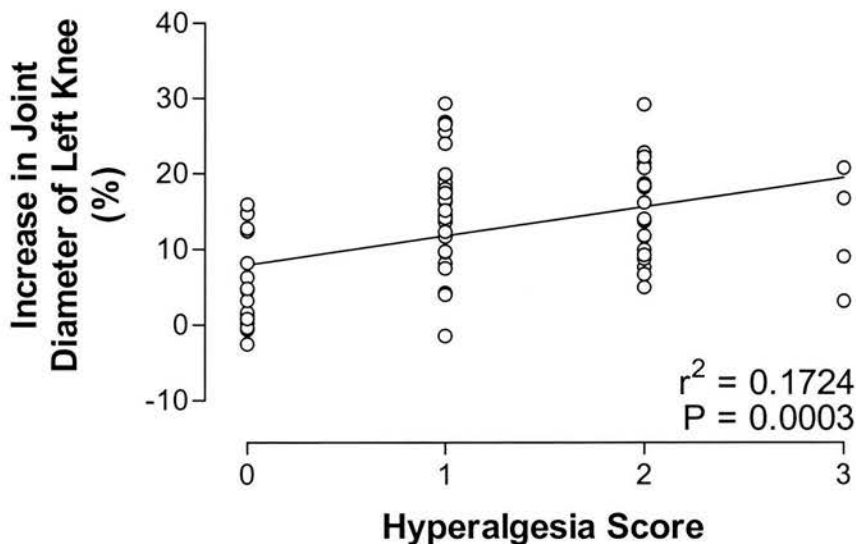


Figure 6.11 – Histopathology of DBA/1 mice receiving repeated intra-articular injections of FCA

Histopathology of a normal (A,B) and arthritic (C-F) knee joints of DBA/1 mice. The normal joint shows an acellular joint space, thin synovial membrane and smooth, healthy cartilage (B; dark blue staining). C) PMNs and macrophages infiltrating the synovium and joint space. D) Thickening of the synovial membrane. E) Peri-articular inflammation of surrounding muscle and ligament. F) Pannus formation causing cartilage destruction and bone erosion. Midline sections stained with H&E (A,C,D,E) or toluidine blue (B,F). *Abbreviations:* S, synovial membrane; JS, joint space; C, cartilage; F, femur; T, tibia; P, patella. Original magnification: A,C – 40X; B,D,E,F – 100x.

Repeated intra-articular injections of FCA produce marked joint and peri articular inflammation resulting in synovial thickening, cartilage destruction, and bone erosion.

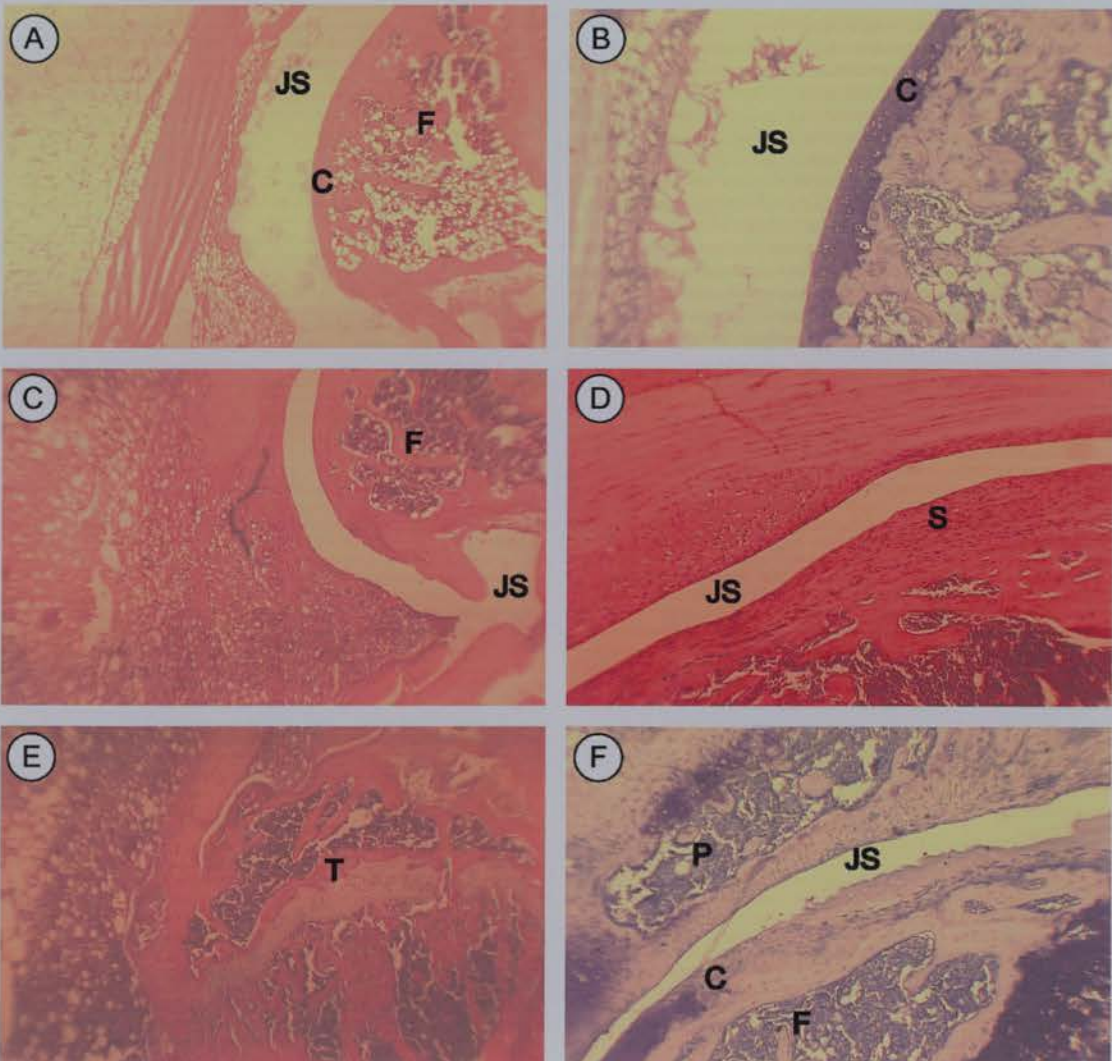


Figure 6.12 – Histopathology of DBA/1 mice receiving repeated intra-articular injections of FCA

Individual histology scores and median (thick line) from left (injected) or right (uninjected) knee joints from DBA/1 mice 7 days after repeated injections (1 per week for 4 weeks) of FCA (5 μ l of a 1 mg ml⁻¹) or HLP (5 μ l). ** P<0.01; *** P <0.001.

Repeated intra-articular injections of FCA induce a chronic inflammation with characteristic arthritic changes. The inflammation is restricted to the injected joint.

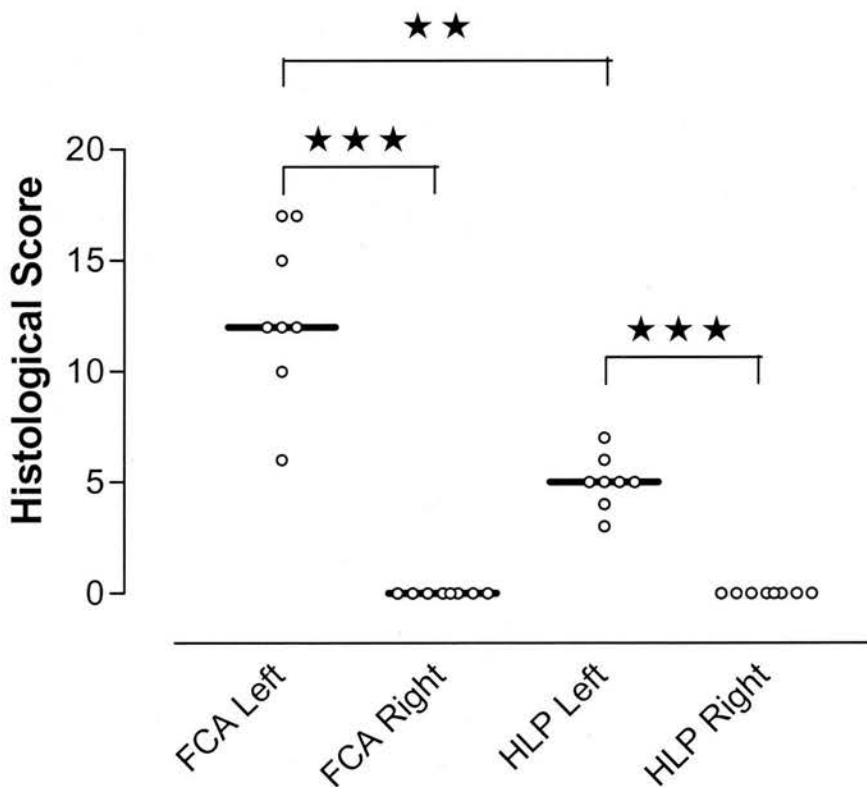


Figure 6.13 – Effect on joint diameter of repeated injections of FCA in the knee of C57BL6 mice

Diameter of A) ipsilateral (left; injected) and B) contralateral (right; uninjected) knee joints of mice receiving repeated intra-articular injections (at arrows; 1 per week for 4 weeks) of FCA (●; 5 μ l of 1 mg ml⁻¹ suspension) or HLP (○; 5 μ l). C) Increase in the left knee diameter as a percentage increase over the right knee diameter, pooled data from comparisons of individual animals. D) Change in the left knee diameter of each animal as compared to the starting value (day 0). Each data point represents the mean \pm SEM.

A chronic inflammation was induced in DBA/1 mice by repeated i.art. injections of FCA.

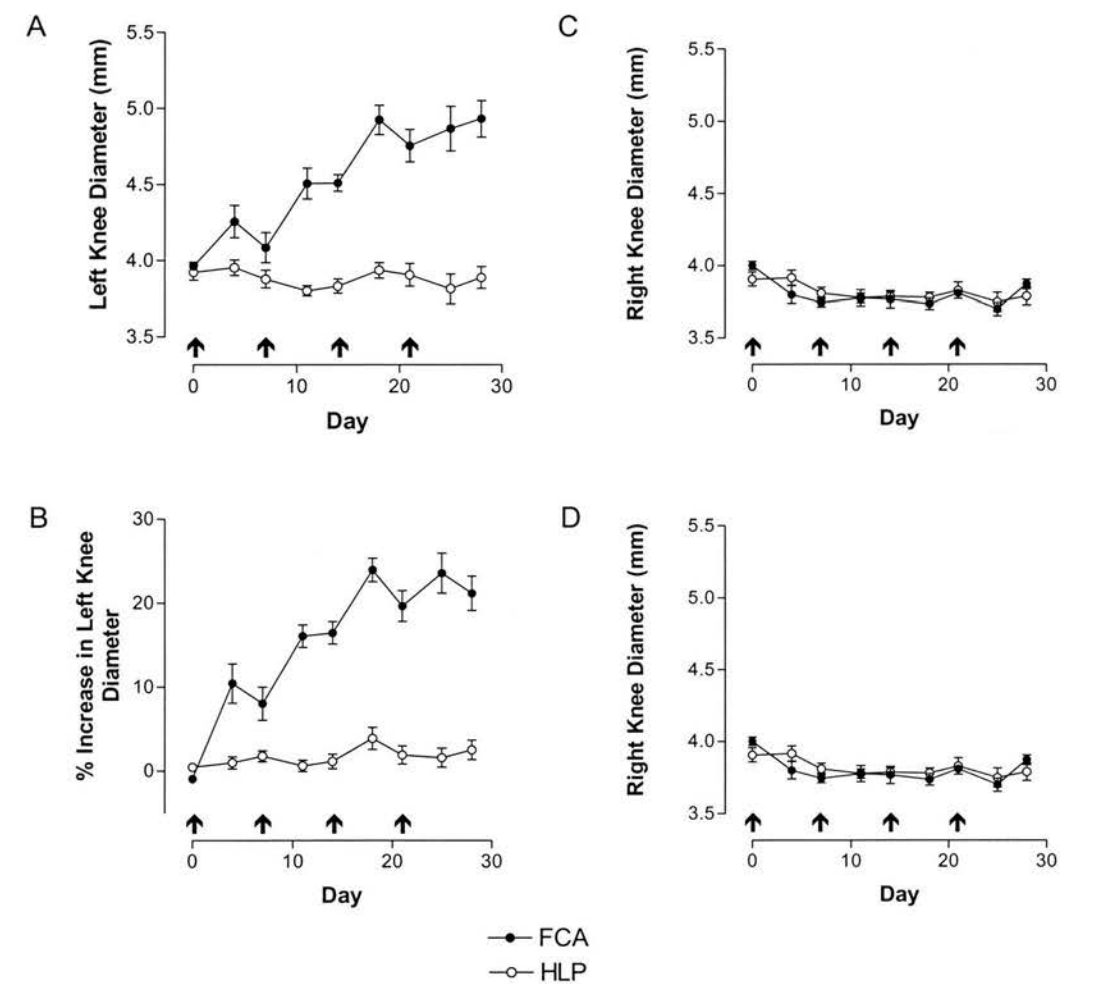


Figure 6.14 – Effect of repeated injections of FCA on body weight in C57BL6 mice

A) Body weight and B) change in body weight from day 0, in C57BL6 mice receiving repeated intra-articular injections (at arrows; 1 per week for 4 weeks) of FCA (●; 5 μ l of 1 mg ml⁻¹ suspension) or HLP (○; 5 μ l). Each data point represents the mean \pm SEM.

Treatment with FCA did not significantly alter the ability of C57BL6 mice to gain weight.

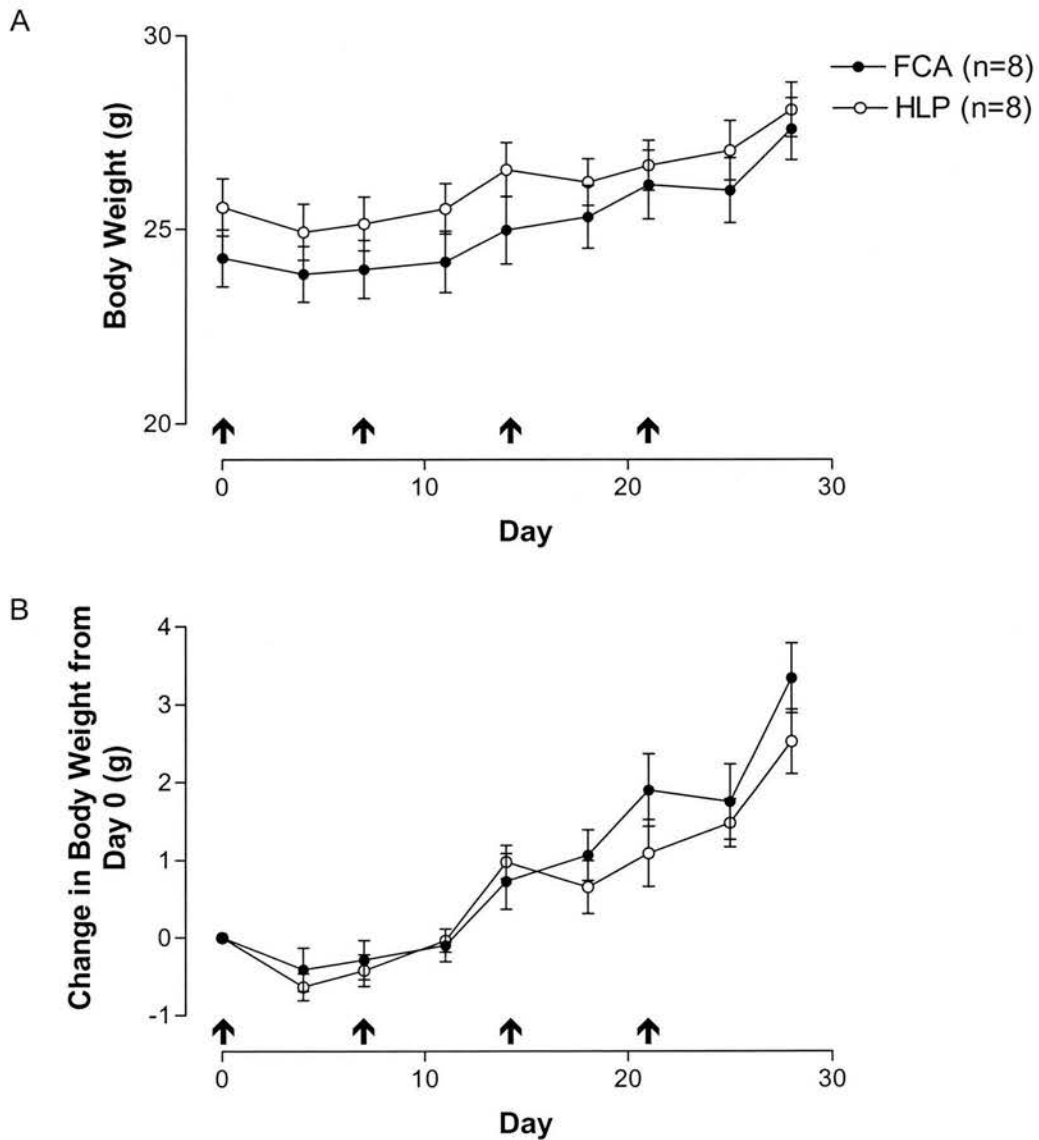
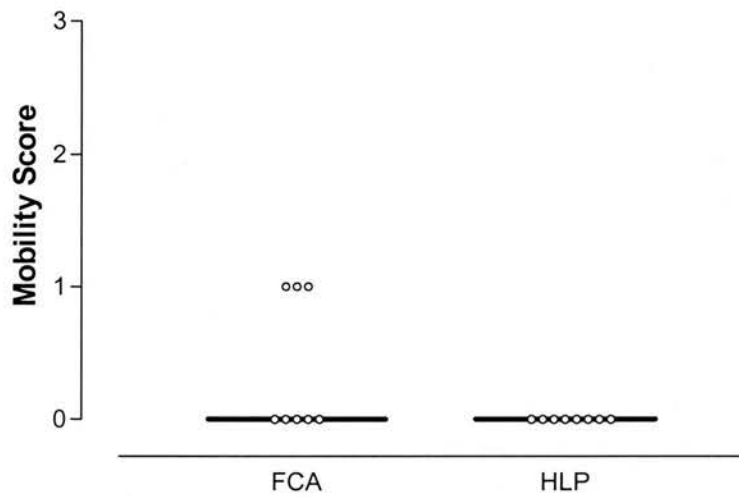


Figure 6.15 - Effect of repeated injections of FCA on mobility in C57BL6 mice

A) Individual scores and median (thick line) of the discomfort associated with walking and B) the correlation of the walking score with the increase in joint diameter in C57BL6 mice 7 days (Day 28) after receiving repeated intra-articular injections (1 per week for 4 weeks) of FCA (5 μ l of 1 mg ml⁻¹ suspension) or HLP (5 μ l).

There was no significant effect on the gait of either group as such no meaningful correlation can be made between walking score and inflammation in the joint.

A



B

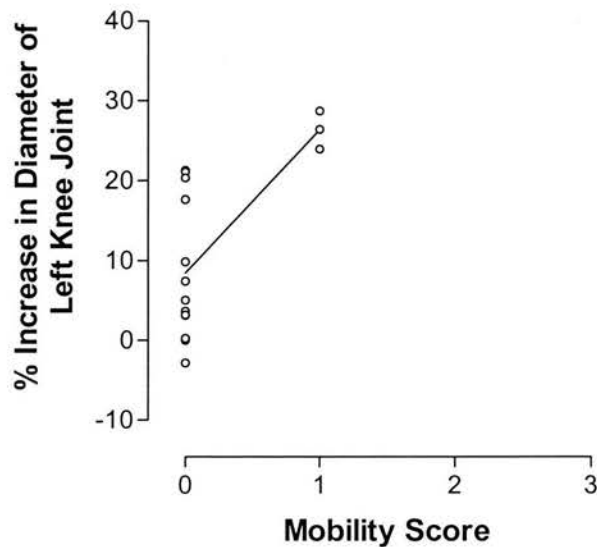


Figure 6.16 – Effect of FCA on hyperalgesia evoked by noxious pressure applied to the knee joint in C57BL6 mice

A) Individual hyperalgesia scores and median (thick line) for pressure applied to the ipsilateral (left; injected) or contralateral (right; uninjected) knee joint in C57BL6 mice 7 days after repeated injections (1 per week for 4 weeks) of FCA (5 μ l of a 1 mg ml⁻¹) or HLP (5 μ l). Joints were subjectively scored on a 0-3 scale (3 maximum) for withdrawal from the noxious stimulus. B) Correlation of the hyperalgesia score and the left knee diameter as a percentage increase over the right knee diameter, pooled data from comparisons of individual animals.

There is a significant increase in the hyperalgesia score for animals that were treated with FCA (** $P = 0.002$, *** $P = 0.0001$; Mann-Whitney). Furthermore there is a significant correlation between the knee joint inflammation and the hyperalgesia score ($P = 0.0001$, Spearman rank correlation).

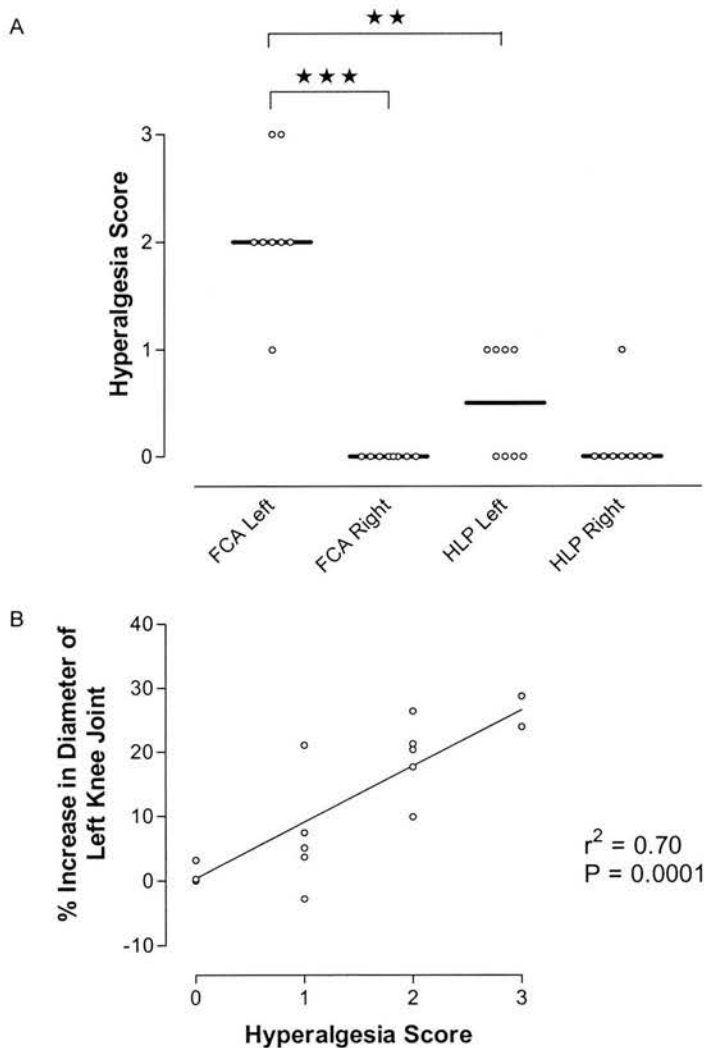


Figure 6.17 – Histopathology of DBA/1 mice receiving repeated intra-articular injections of FCA

Histopathology of a normal (A,B) and arthritic (C-F) knee joints of C57BL/6 mice. The normal joint shows an acellular joint space, thin synovial membrane and smooth, healthy cartilage (B; dark blue staining). C) PMNs and macrophages infiltrating the synovium and joint space. D) Pannus formation overgrowing articular cartilage and the joint space contains many inflammatory cells and cell debris. E) Thickening of the synovial membrane and the formation of sparsely packed bone. F) Pannus formation causing cartilage destruction and bone erosion. Midline sections stained with H&E (C,D,E) or toluidine blue (A,B,F). Abbreviations: S, synovial membrane; JS, joint space; C, cartilage; F, femur. Original magnification: A,C – 40X; B,D,E,F – 100x.

Repeated intra-articular injections of FCA produce marked joint and peri articular inflammation resulting in synovial thickening, cartilage destruction, and bone erosion/formation.

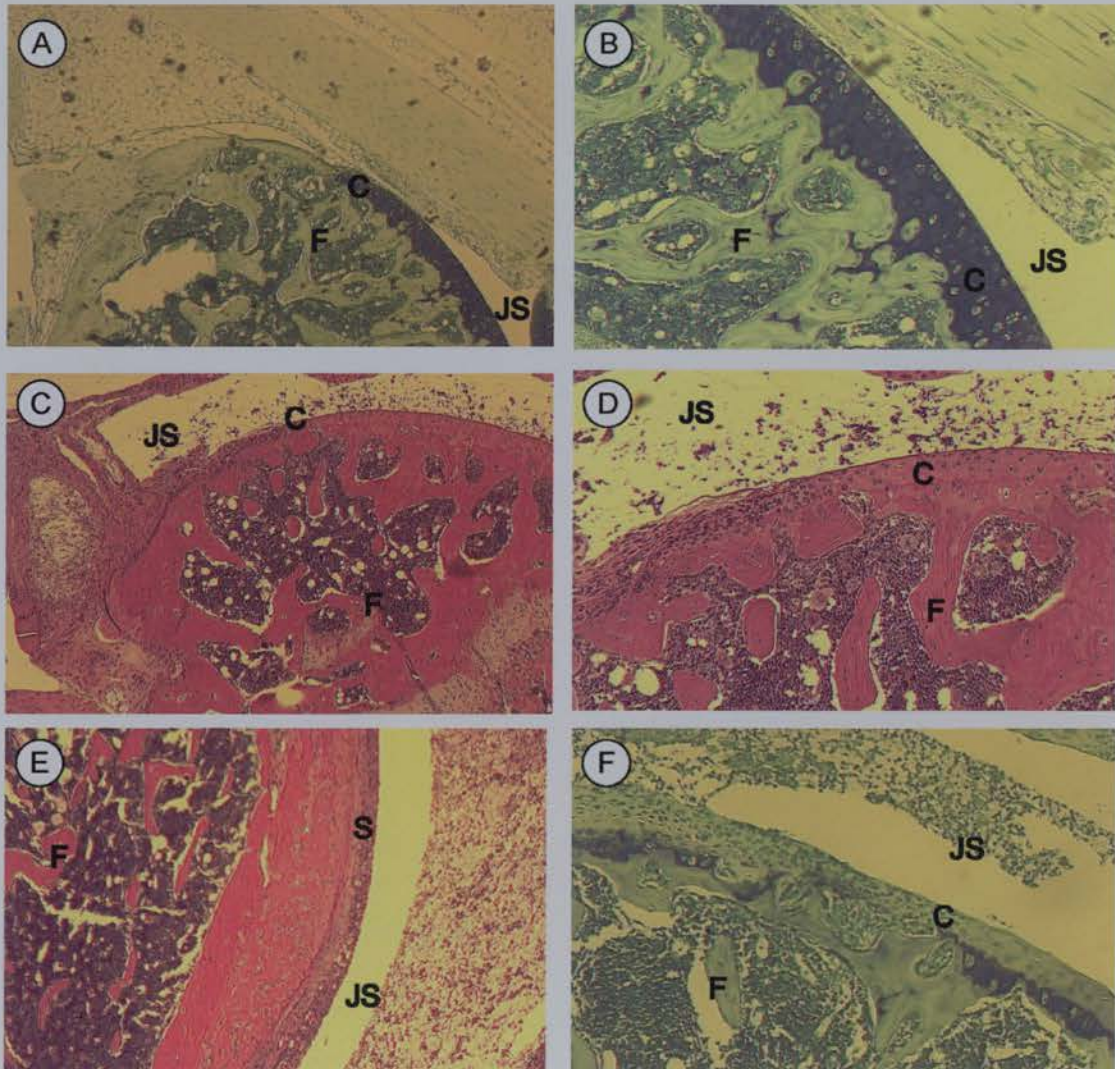


Figure 6.18 – Histopathology of C57BL6 mice receiving repeated intra-articular injections of FCA

Individual histology scores and median (thick line) from left (injected) or right (uninjected) knee joints from C57BL6 mice 7 days after repeated injections (1 per week for 4 weeks) of FCA (5 μ l of a 1 mg ml⁻¹) or HLP (5 μ l). * P<0.05; ** P<0.01; *** P <0.001.

Repeated intra-articular injections of FCA induce a chronic inflammation with characteristic arthritic changes. The inflammation is restricted to the injected joint.

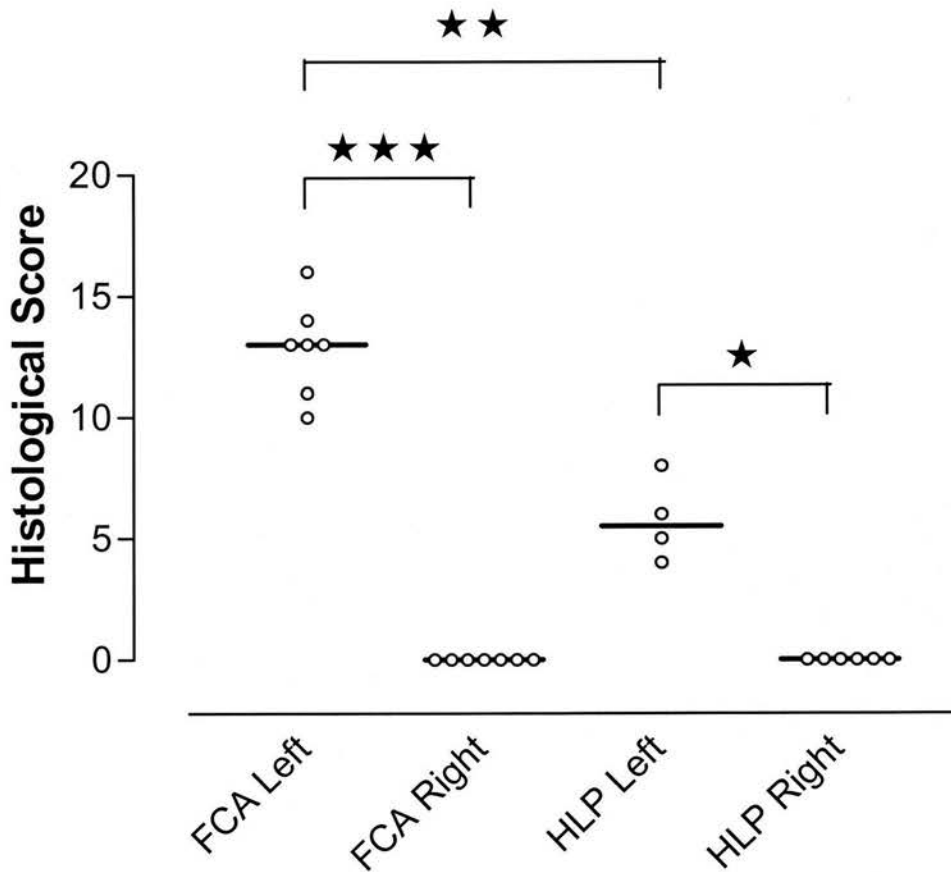


Figure 6.19 – Comparison of knee diameter during FCA-induced unilateral arthritis in DBA/1 and C57BL6 mice

Increase in the left knee diameter as a percentage increase over the right knee diameter in DBA/1 (■) and C57BL6 (●) mice receiving repeated intra-articular injections of FCA (at arrows; 1 per week for 4 weeks; 5 μ l of 1 mg ml⁻¹ suspension). Each data point represents the mean \pm SEM.

There was no significant difference between DBA/1 and C57BL6 mice in the ability of FCA to induce a chronic unilateral inflammation.

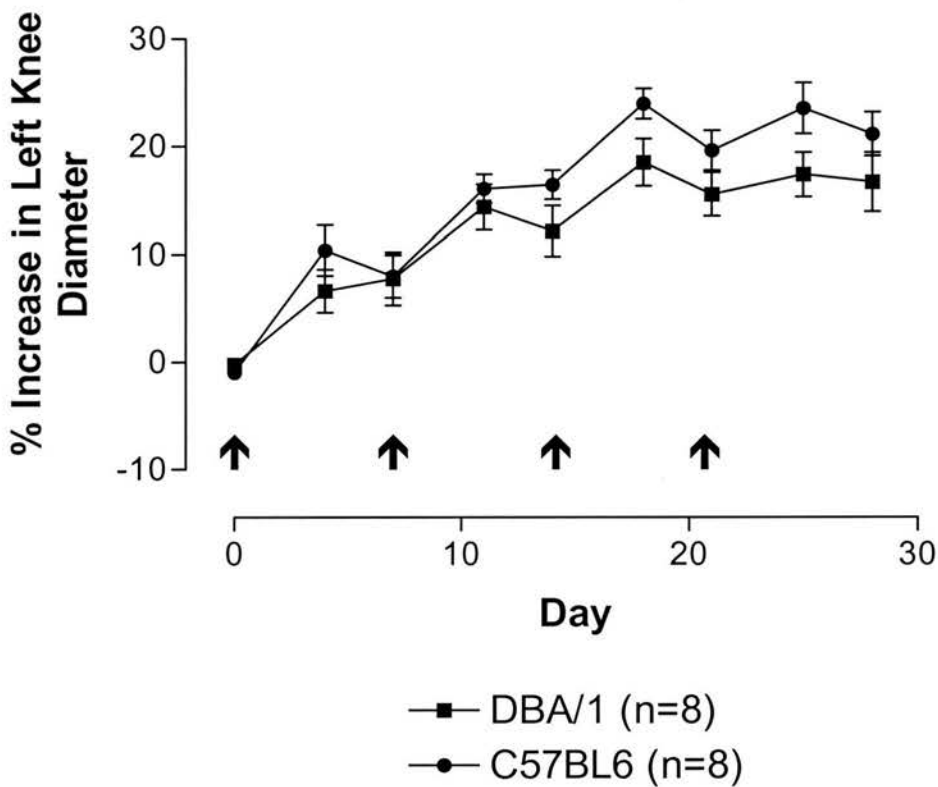


Figure 6.20 – Comparison of mobility during FCA-induced unilateral arthritis in DBA/1 and C57BL6 mice

Individual mobility scores and median (thick line) in DBA/1 and C57BL6 mice 7 days after receiving repeated intra-articular injections of FCA (1 per week for 4 weeks; 5 μ l of 1 mg ml⁻¹ suspension).

Neither strain showed any alteration in gait during chronic unilateral inflammation evoked by FCA

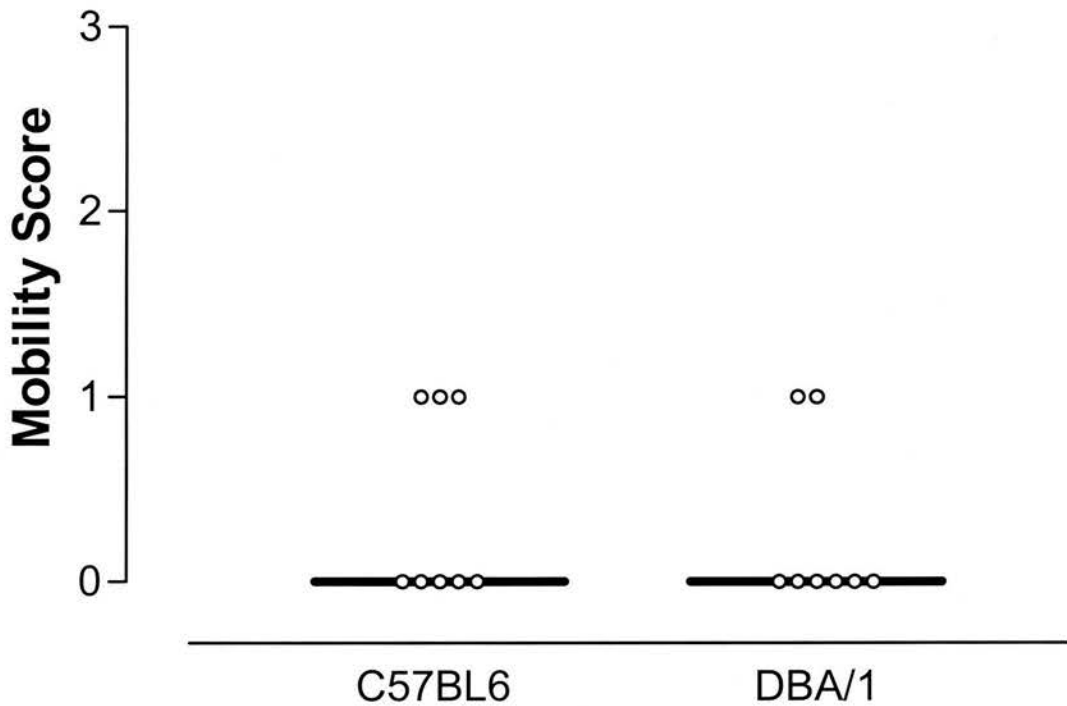


Figure 6.21 – Comparison of hyperalgesia evoked by noxious pressure applied to the knee joint in DBA/1 and C57BL6 mice

Hyperalgesia score and median (thick line) of the left (chronically inflamed) and right (control) knee joints in DBA/1 and C57BL6 mice 7 days after receiving repeated intra-articular injections (1 per week for 4 weeks) of FCA (5 μ l of 1 mg ml⁻¹ suspension). * $P < 0.05$, *** $P < 0.0001$ (Mann-Whitney).

Both strains showed a significant sensitisation to noxious pressure on the inflamed joint when compared to the contralateral control. There was no difference between strains in the withdrawal response.

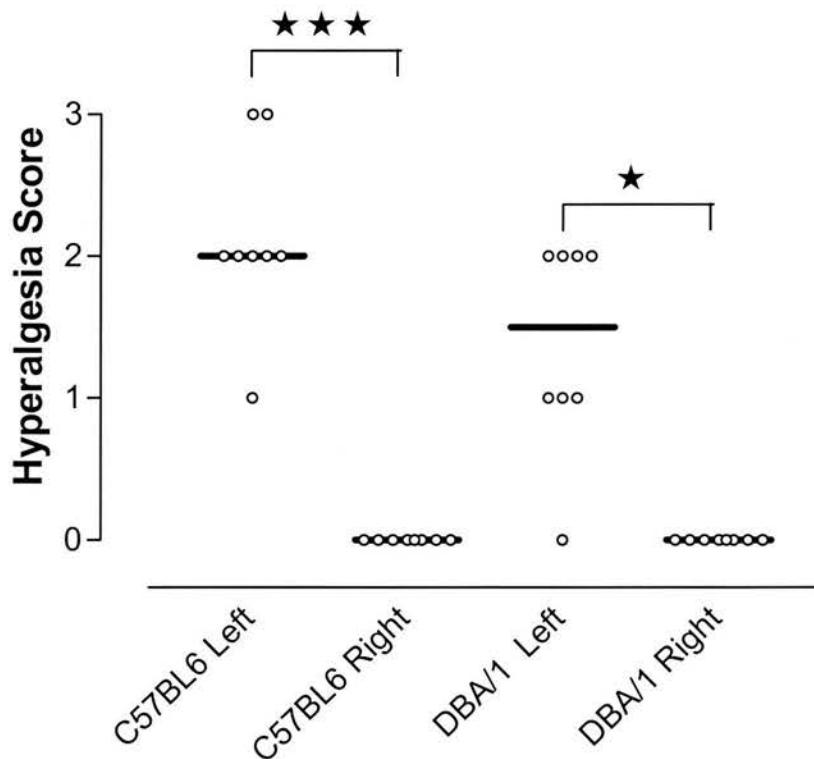


Figure 6.22 – Comparison of histological scores in DBA/1 and C57BL6 mice receiving repeated intra-articular injections of FCA

Individual histology scores and median (thick line) from left (injected) or right (uninjected) knee joints from DBA/1 and C57BL6 mice 7 days after repeated injections (1 per week for 4 weeks) of FCA ($5\ \mu\text{l}$ of a $1\ \text{mg ml}^{-1}$) or HLP ($5\ \mu\text{l}$). *** $P < 0.001$.

There is no significant difference in the histological score of pathological changes resulting from FCA-induced chronic inflammation between DBA/1 and C57BL6 mice.

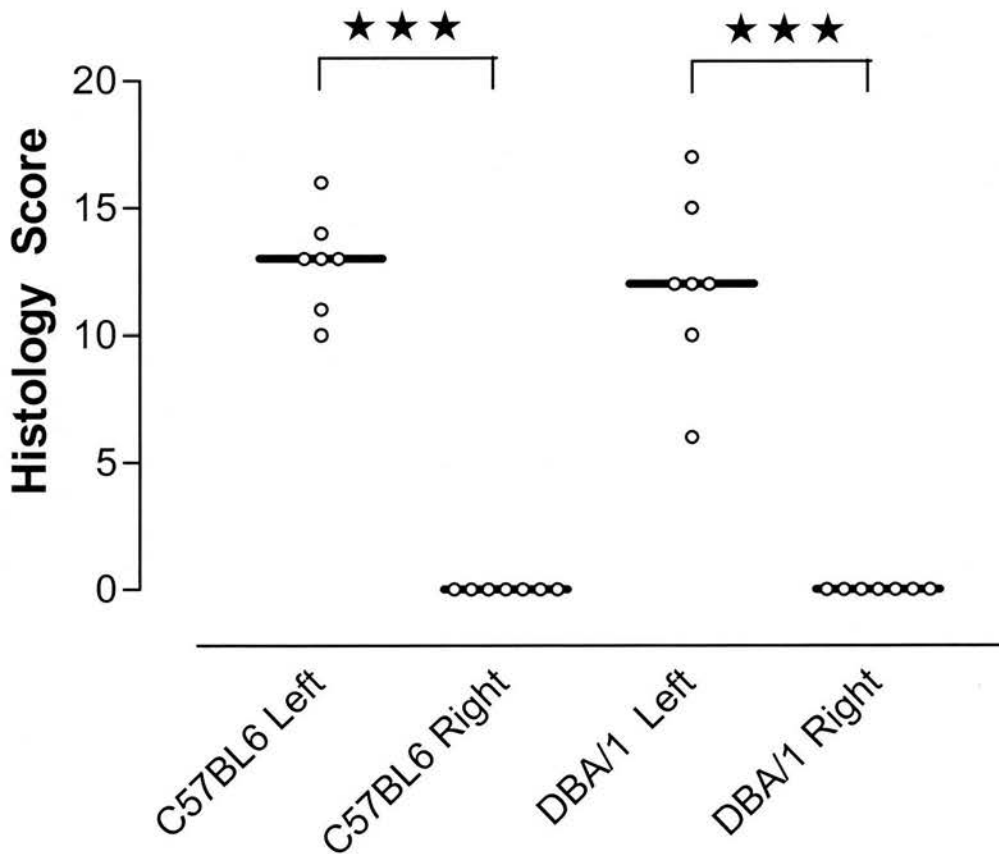


Figure 6.23 – Effect of prednisolone on knee diameter during established FCA induced unilateral arthritis in DBA/1 mice

Diameter of A) left (chronically inflamed) and B) right (control) knee joints of mice receiving repeated intra-articular injections (at arrows; 1 per week for 4 weeks) of FCA (5 μ l of 1 mg ml⁻¹ suspension) and a 7 day treatment (thick bar; daily i.p. injection) with prednisolone (●; 10 mg kg⁻¹) or injection of vehicle (○) starting from Day 28. C) Increase in the left knee diameter as a percentage increase over the right knee diameter, pooled data from comparisons of individual animals. D) Change in the left knee diameter of each animal as compared to the starting value (day 0). Each data point represents the mean \pm SEM.

Treatment with prednisolone reduced the swelling in chronically inflamed joints.

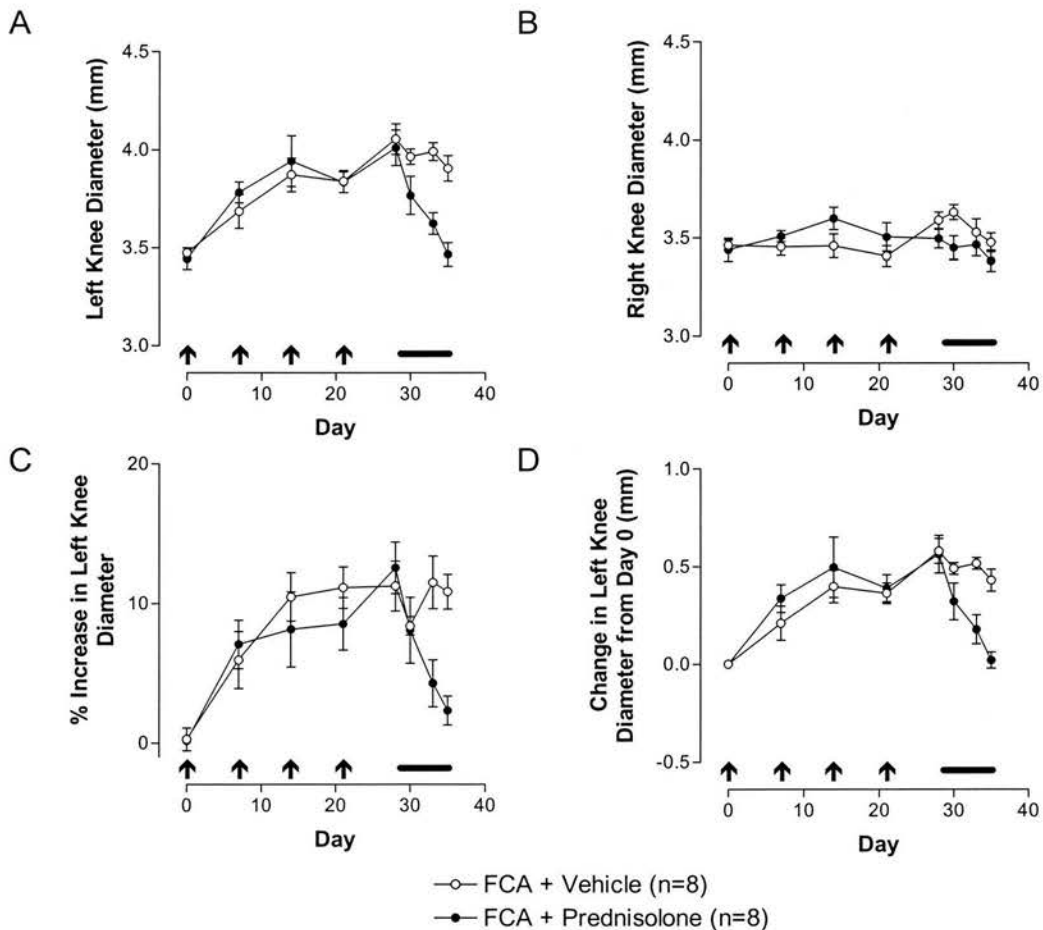


Figure 6.24 – Effect of prednisolone on body weight during established FCA-induced unilateral arthritis in DBA/1 mice

A) Body weight and B) change in body weight from Day 0, of mice receiving repeated intra-articular injections of FCA (at arrows; 1 per week for 4 weeks; 5 μl of 1 mg ml^{-1} suspension) and a 7 day treatment (thick bar; daily i.p. injection) with prednisolone (●; 10 mg kg^{-1}) or injection vehicle (○) starting from Day 28. Each data point represents the mean \pm SEM.

Treatment with prednisolone did not affect weight gain.

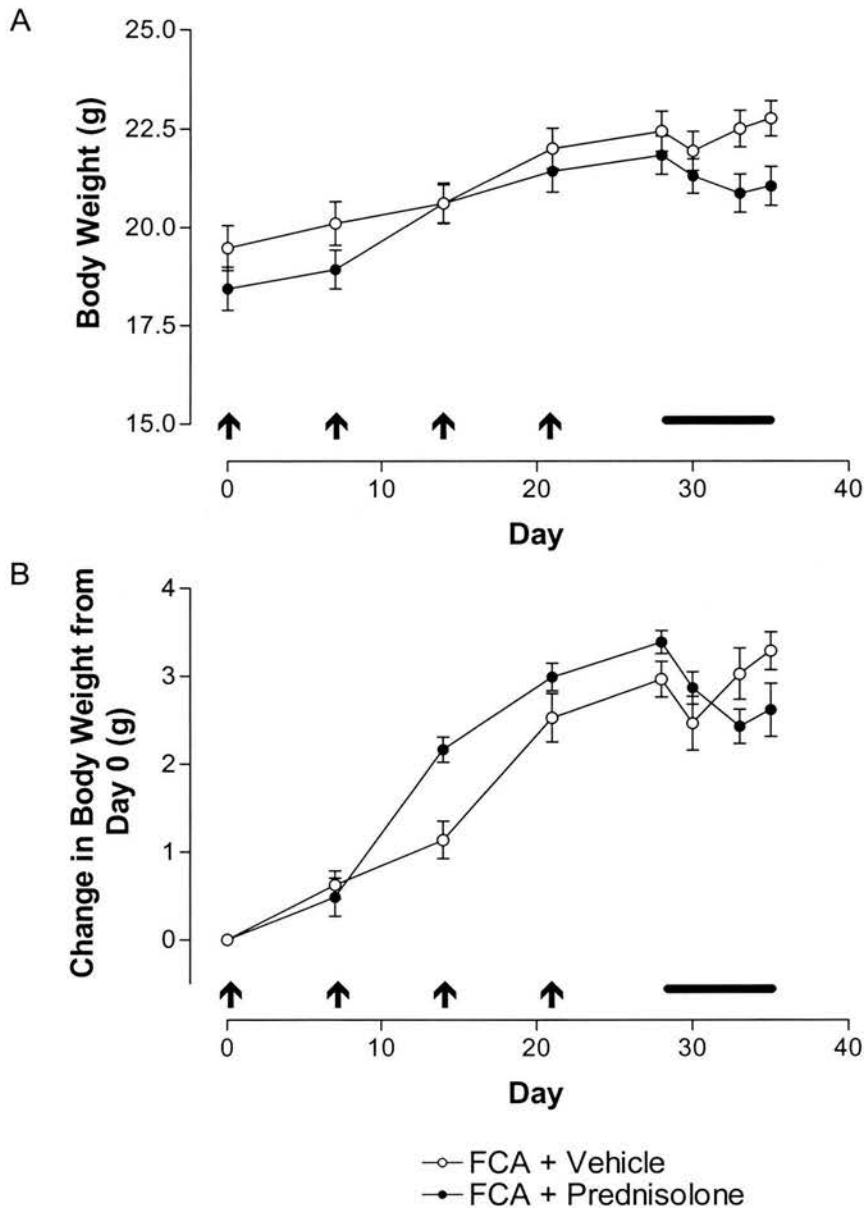


Figure 6.27 – Effect of prednisolone on inflammation and joint destruction during established FCA-induced unilateral arthritis in DBA/1 mice.

Histopathology of arthritic knee joints following 7 days treatment with prednisolone (10 mg kg^{-1} , daily i.p. injections) in DBA/1 mice. The normal joint shows an acellular joint space, thin synovial membrane and smooth, healthy cartilage (B; dark blue staining). A) Infiltration of a large number of PMNs and macrophages into the synovial membrane, peri-articular tissues, and joint space. B) Pannus formation and the destruction of articular cartilage. Midline sections stained with A) H&E or B) toluidine blue. *Abbreviations:* JS, joint space; C, cartilage; F, femur. Original magnification: A – 40X; B – 100x.

Treatment with prednisolone did not significantly reverse arthritic changes in established FCA-induced chronic inflammation.

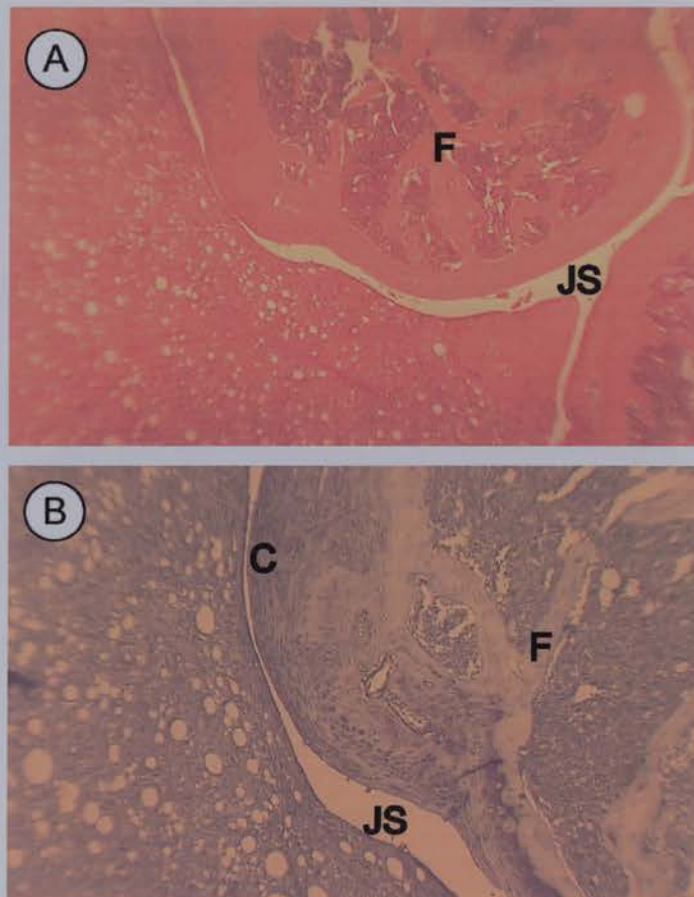


Figure 6.28 – Effect of prednisolone on the histological score during established FCA-induced unilateral arthritis in DBA/1 mice

Individual histological score and median (thick line) for the left (chronically inflamed) and right (control) knee joint 7 days after treatment with prednisolone (10 mg kg^{-1}) or vehicle (daily i.p. injections) in DBA/1 mice. *** $P < 0.001$.

Treatment with prednisolone did not alter the joint destruction in a chronically inflamed joint.

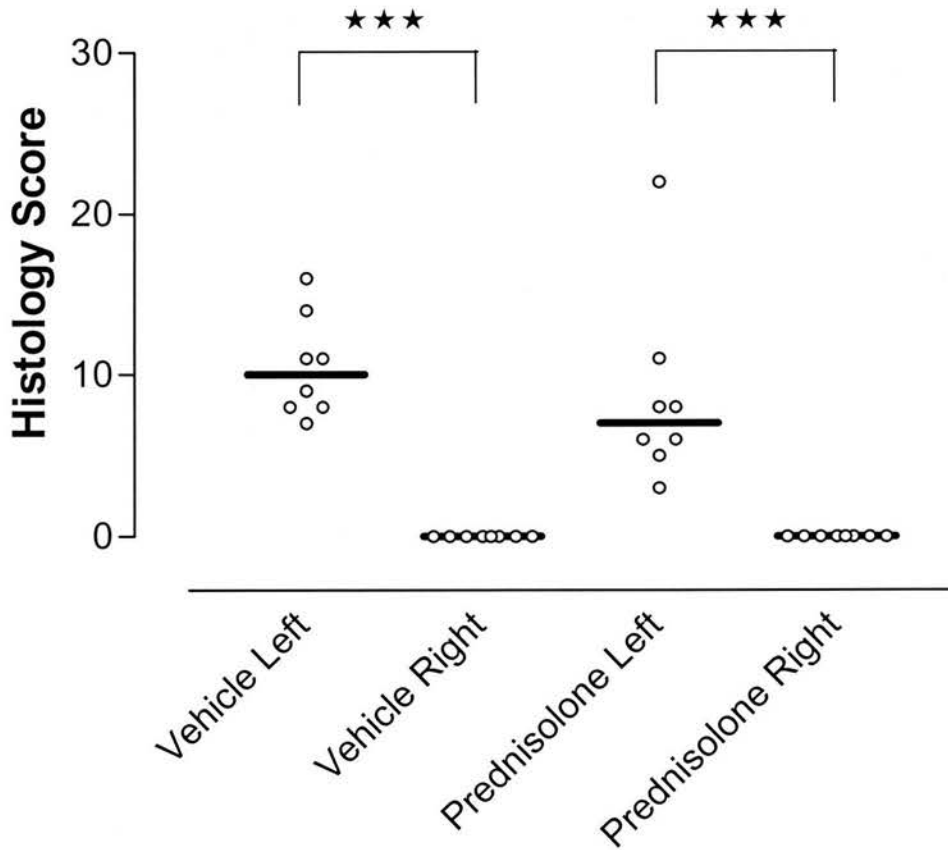


Figure 6.29 – Effect of indomethacin on knee diameter during established FCA-induced unilateral arthritis in C57BL6 mice

Diameter of A) left (chronically inflamed) and B) right (control) knee joints of mice receiving repeated intra-articular injections of FCA (at arrows; 1 per week for 4 weeks; 5 μl of 1 mg ml^{-1} suspension) and a 7 day treatment (thick bar; daily s.c. injection) with indomethacin (●; 1 mg kg^{-1}) or injection vehicle (○) starting from Day 28. C) Increase in the left knee diameter as a percentage increase over the right knee diameter, pooled data from comparisons of individual animals. D) Change in the left knee diameter of each animal as compared to the starting value (day 0). Each data point represents the mean \pm SEM.

Treatment with indomethacin reduced the swelling present in chronically inflamed joints.

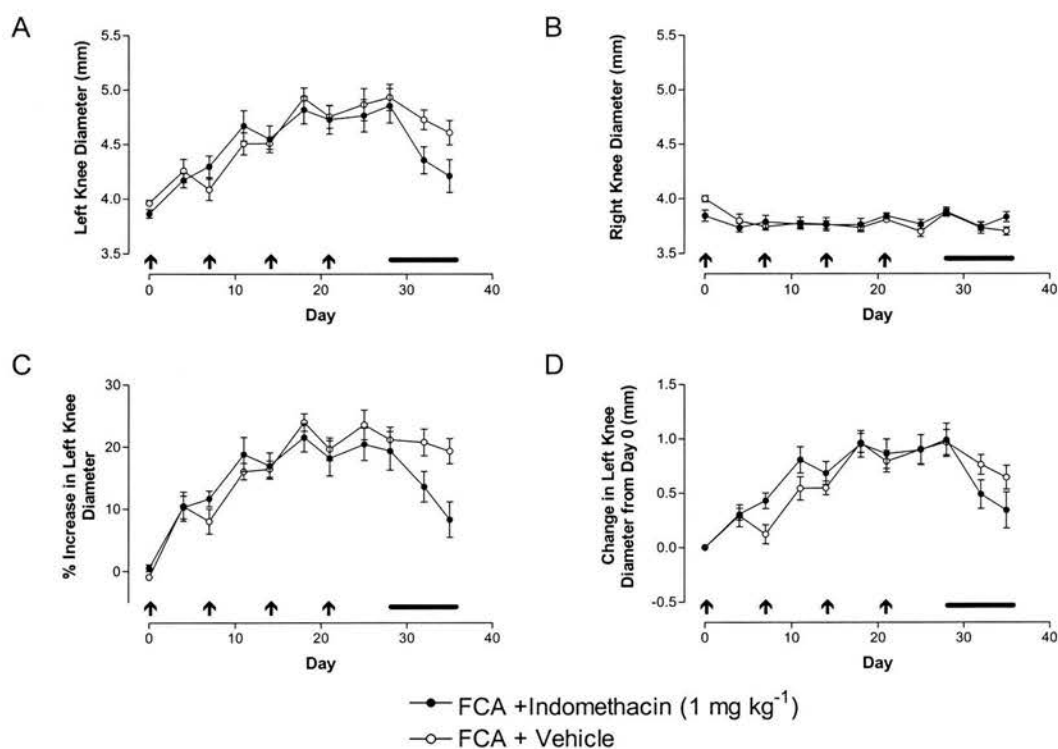


Figure 6.30 – Effect of indomethacin on body weight during established FCA-induced unilateral arthritis in C57BL6 mice

A) Body weight and B) change in body weight from Day 0, of mice receiving repeated intra-articular injections of FCA (at arrows; 1 per week for 4 weeks; 5 μl of 1 mg ml^{-1} suspension) and a 7 day treatment (thick bar; daily s.c. injection) with indomethacin (●; 1 mg kg^{-1}) or injection vehicle (○) starting from Day 28. Each data point represents the mean \pm SEM.

Treatment with indomethacin did not affect weight gain.

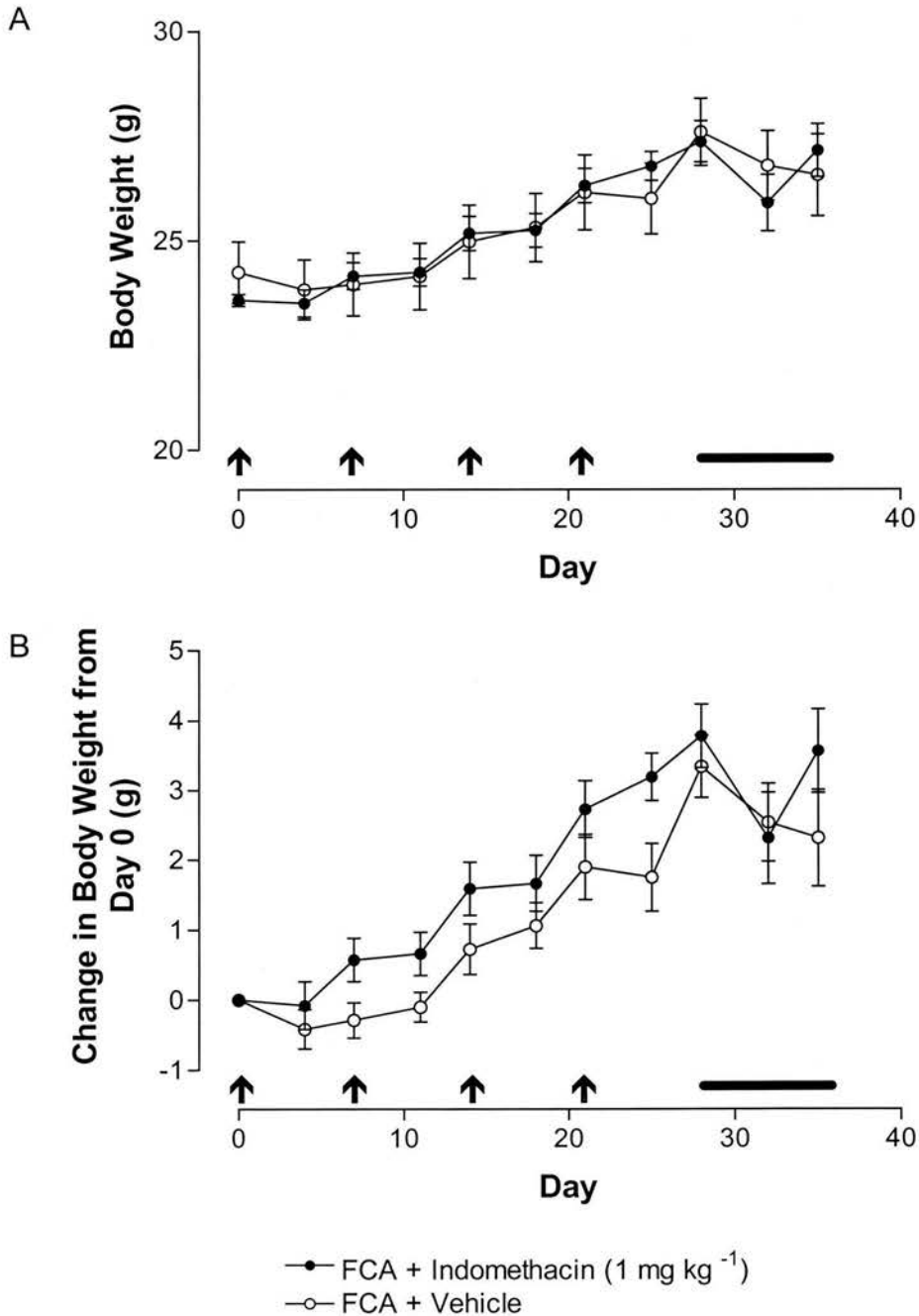


Figure 6.32 – Effect of indomethacin on hyperalgesia evoked by noxious pressure during established FCA-induced unilateral arthritis in C57BL6 mice

Individual hyperalgesia scores and median (thick line) for pressure applied to the left (chronically inflamed) or right (control) A) during chronic inflammation at Day 28 (pre-treatment) or B) after 7 days treatment with indomethacin (1 mg kg^{-1}) or vehicle (daily s.c. injections) in C57BL6 mice. * $P < 0.05$, *** $P < 0.001$.

Treatment with indomethacin significantly decreased the sensitisation to a noxious stimulus in chronically inflamed joints.

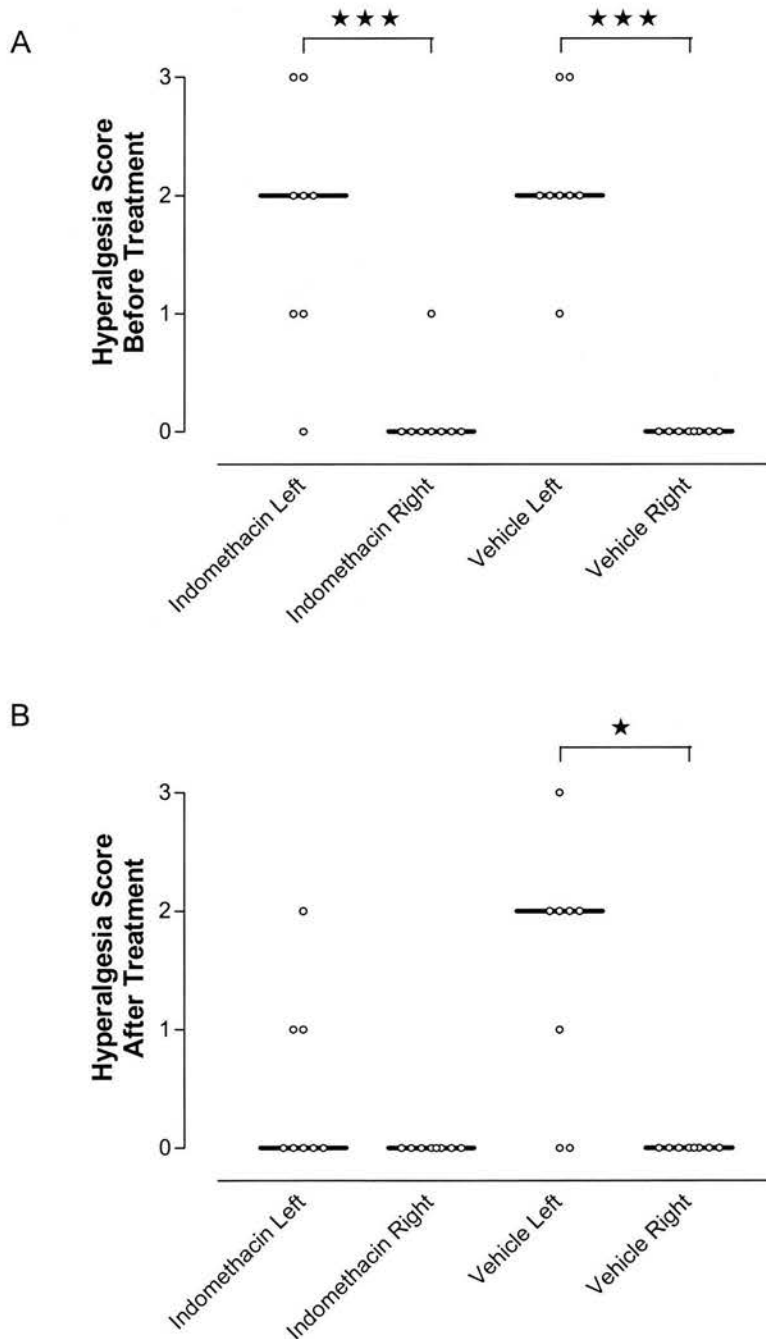


Figure 6.33 – Effect of indomethacin on inflammation and joint destruction in established FCA-induced unilateral arthritis in C57BL6 mice.

Histopathology of arthritic knee joints following 7 days treatment with indomethacin (1 mg kg^{-1} , daily i.p. injections) in C57BL6 mice. The normal joint shows an acellular joint space, thin synovial membrane and smooth, healthy cartilage (B; dark blue staining). A) Extensive peri-articular inflammation and infiltration of PMN cells into the joint space. B) Destruction of articular cartilage and inflammation of the patella tendon. Midline sections stained with A) H&E or B) toluidine blue. *Abbreviations:* JS, joint space; C, cartilage; F, femur; PT, patella tendon. Original magnification: A – 40X; B – 100x.

Treatment with indomethacin did not significantly reverse arthritic changes in established FCA-induced chronic inflammation.

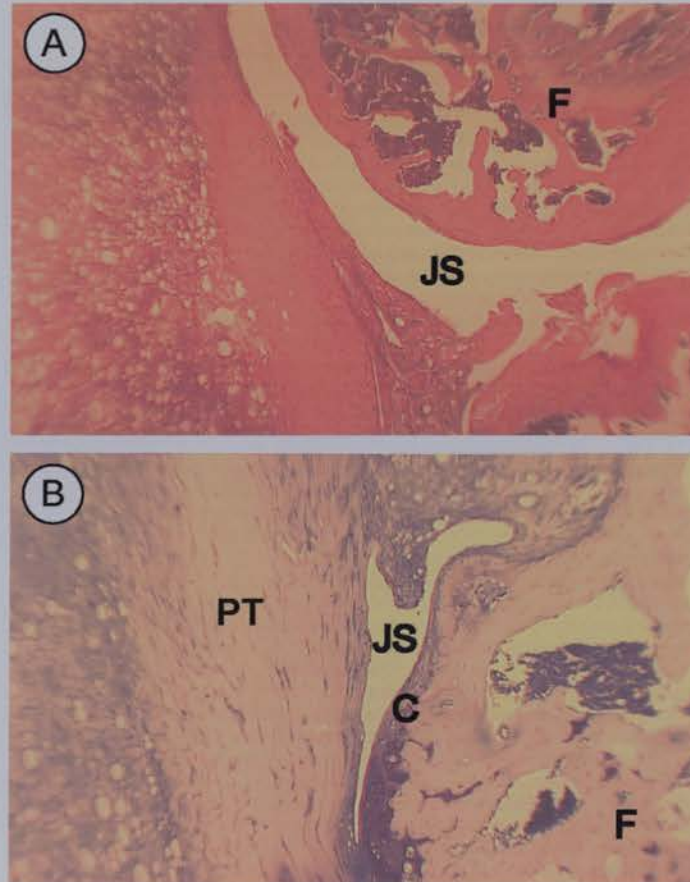


Figure 6.34 – Effect of indomethacin on the histological score in established FCA-induced unilateral arthritis in C57BL6 mice

Histological score and median (thick line) for the left (chronically inflamed) or right (control) knee joint 7 days after treatment with indomethacin (1 mg kg^{-1}) or vehicle (daily s.c. injections) in C57BL6 mice. *** $P < 0.001$ (Mann-Whitney).

Treatment with indomethacin did not alter the pathological changes and joint destruction in chronically inflamed joints.

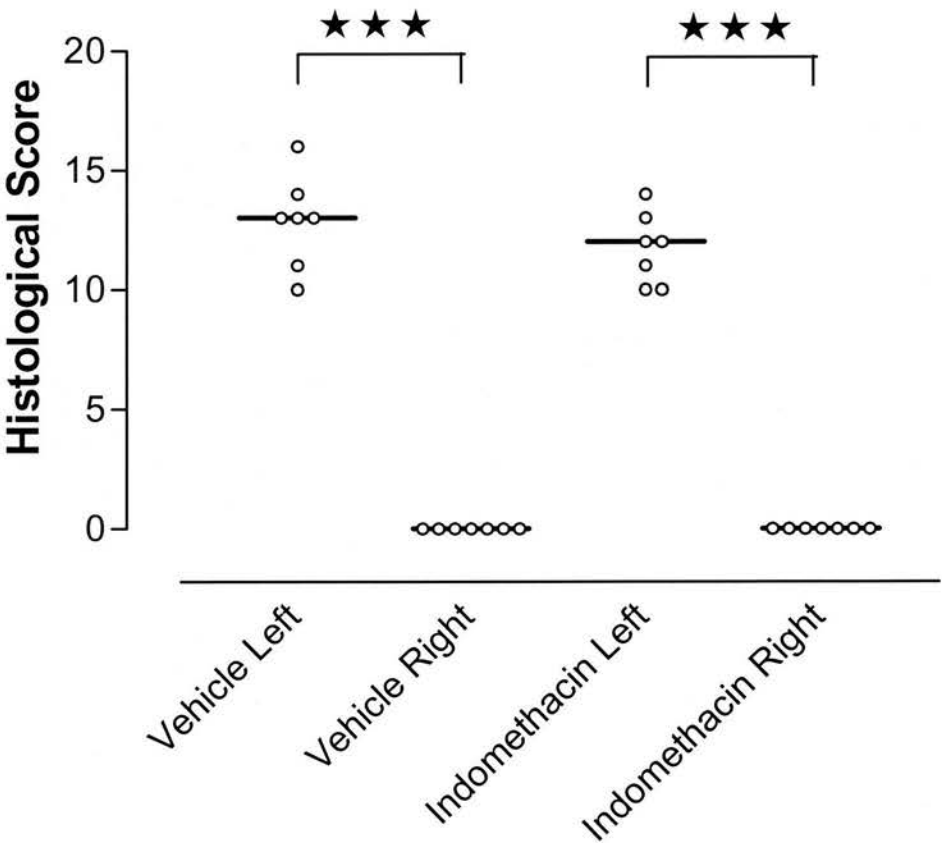


Figure 6.35 – Effect of FCA-induced unilateral arthritis on knee diameter in P2X7 knock out mice.

Diameter of A) ipsilateral (left) and B) contralateral (right) knee joints of P2X7 knock out transgenic mice (TG; circles) or wild type littermates (WT; squares) on a C57BL6 background receiving repeated intra-articular injections (at arrows; 1 per week for 4 weeks) of FCA (filled symbols; 5 μ l of 1 mg ml⁻¹ suspension) or HLP (empty symbols; 5 μ l). C) Increase in the left knee diameter as a percentage increase over the right knee diameter, pooled data from comparisons of individual animals. D) Change in the left knee diameter of each animal as compared to the starting value (day 0). Each data point represents the mean \pm SEM.

There was no significant difference in the ability to induced arthritis between KO and WT mice.

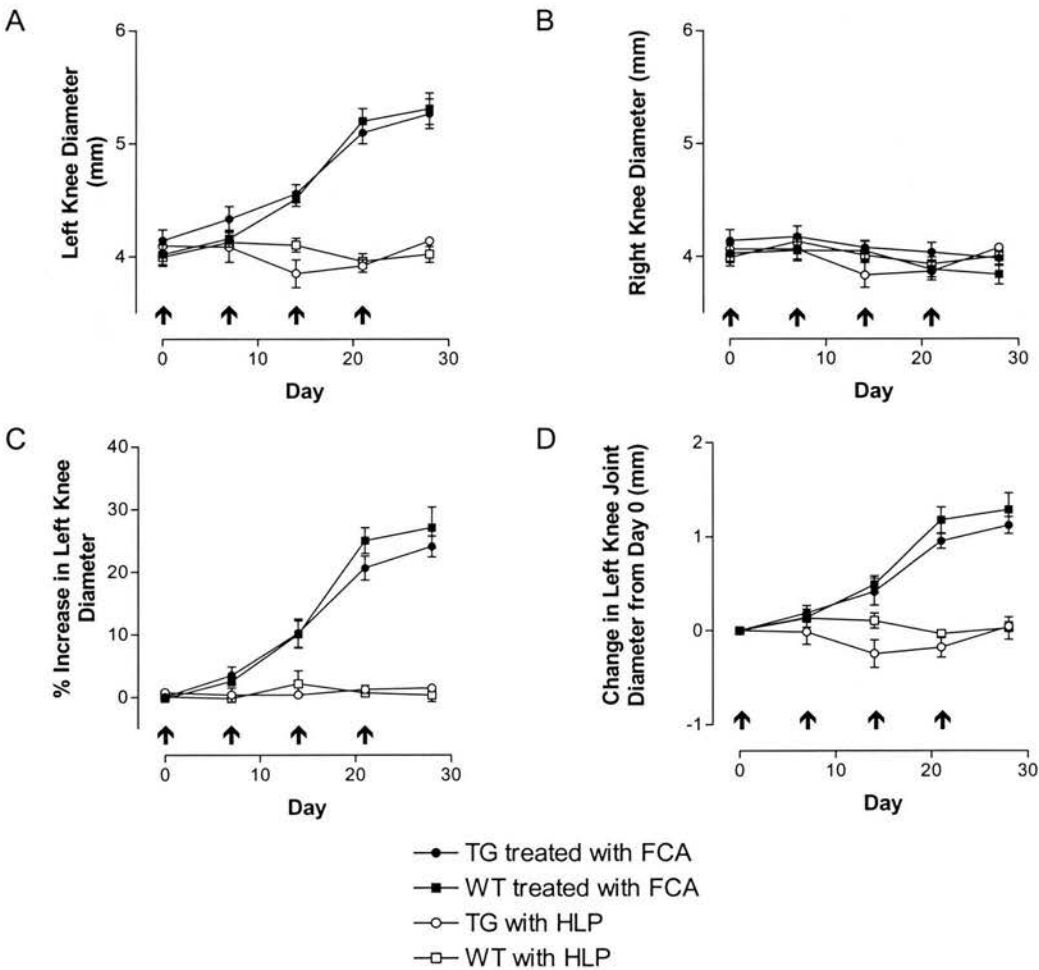


Figure 6.36 – Effect of FCA on body weight in P2X7 KO and WT mice.

A) Body weight and B) Change in body weight from day 0, of P2X7 knock out transgenic mice (KO; circles) or wild type littermates (WT; squares) on a C57BL6 background receiving repeated intra-articular injections (at arrows; 1 per week for 4 weeks) of FCA (filled symbols; 5 μ l of 1 mg ml⁻¹ suspension) or HLP (empty symbols; 5 μ l). Each data point represents the mean \pm SEM.

There was a significant difference between FCA and HLP treated animals ($P=0.02$ and $P=0.004$ for WT and KO respectively) but not between KO and WT animals receiving either treatment ($P=0.59$ and $P=0.49$ for FCA and HLP respectively).

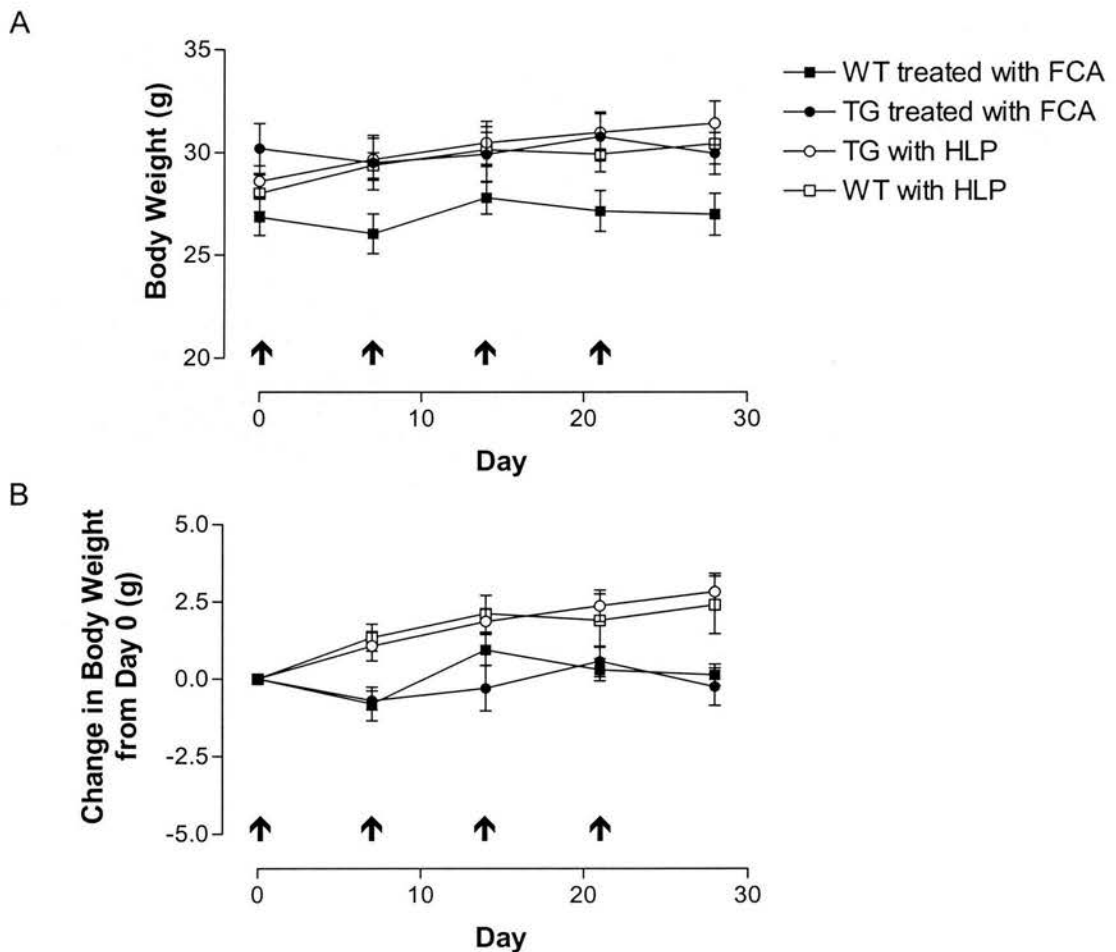


Figure 6.37 – Effect of repeated injections of FCA on mobility in P2X7 KO and WT mice

Individual mobility score and median (thick line) of the discomfort associated with walking in P2X7 knock out transgenic mice (KO) or wild type littermates (WT) on a C57BL6 background 7 days (Day 28) after receiving repeated intra-articular injections (1 per week for 4 weeks) of FCA (5 μ l of 1 mg ml⁻¹ suspension) or HLP (5 μ l).

Animals treated with FCA showed discomfort as manifest in limping. This was not seen in HLP treated animals. There was no significant difference in the scoring between KO and WT groups ($P = 0.59$; unpaired t-test).

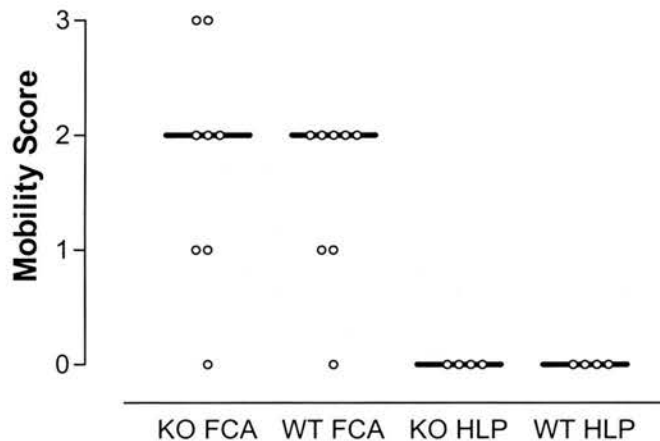


Figure 6.38 - Effect of repeated injections of FCA on hyperalgesia evoked by noxious pressure in P2X7 KO and WT mice

Individual hyperalgesia scores and median (thick line) of the hyperalgesia produced by squeezing the knee joint in P2X7 knock out transgenic mice (KO) or wild type littermates (WT) on a C57BL6 background 7 days (Day 28) after receiving repeated intra-articular injections (1 per week for 4 weeks) of FCA (5 μ l of 1 mg ml⁻¹ suspension) or HLP (5 μ l) in the left knee joint.

Joints from both KO and WT mice that were injected with FCA showed a sensitisation to a painful stimulus when compared to the contralateral limb ($P = 0.0002$ and $P = 0.0002$ respectively; paired t-test) or to vehicle controls ($P = 0.004$ and $P = 0.01$ respectively; Mann-Whitney). There was no significant difference in the scoring between the injected knee joint (left) of KO and WT groups that received FCA ($P > 0.99$; unpaired t-test).

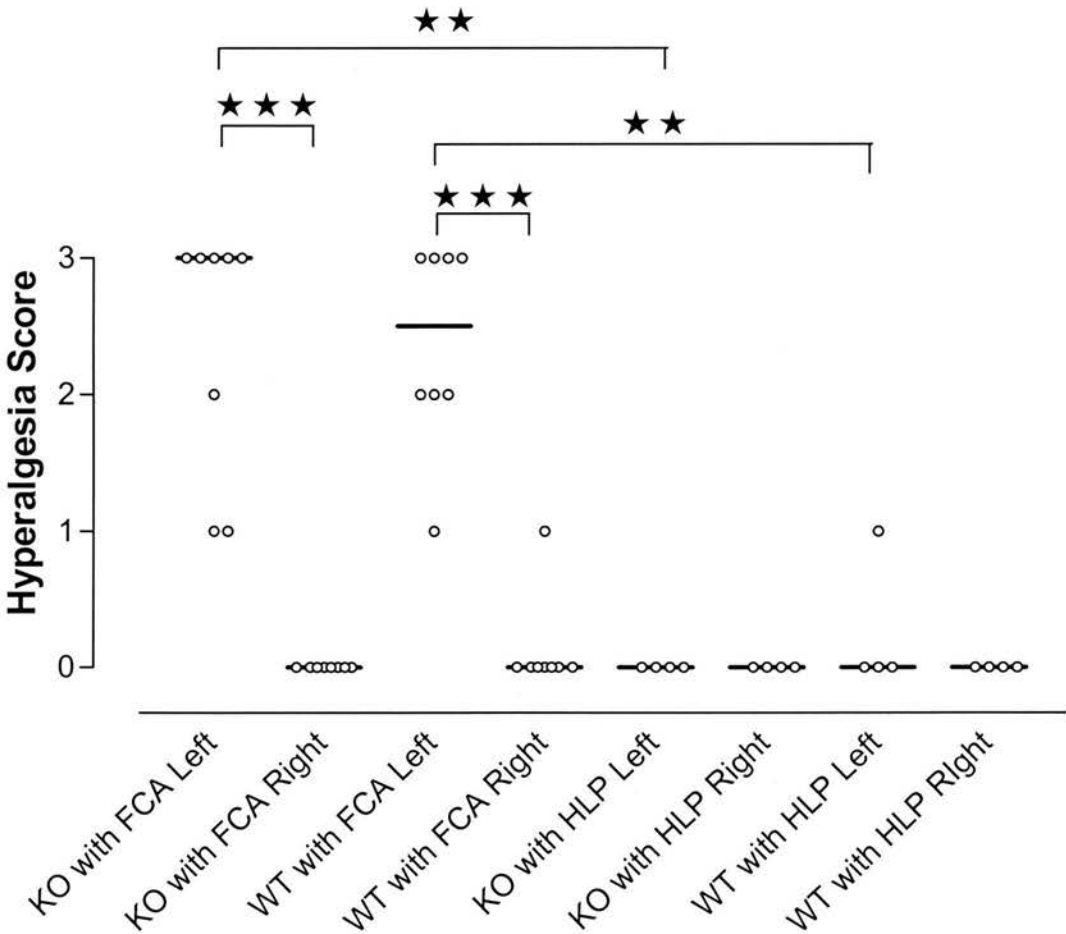


Figure 6.39 - Effect of FCA on the histological score in P2X7 KO and WT mice

Individual histological scores and median (thick line) of joint damage in knee joint sections of P2X7 knock out transgenic mice (KO) or wild type littermates (WT) on a C57BL6 background 7 days (Day 28) after receiving repeated intra-articular injections (1 per week for 4 weeks) of FCA (5 μ l of 1 mg ml⁻¹ suspension) or HLP (5 μ l) in the left knee joint.

Joints from both KO and WT mice that were injected with FCA showed significant joint damage when compared to the contralateral limb ($P = 0.0001$ and $P = 0.0002$ respectively; paired t-test) or to vehicle controls ($P = 0.004$ and $P = 0.008$ respectively; Mann-Whitney). There was no significant difference in the scoring between the injected knee joint (left) of KO and WT groups that received FCA ($P = 0.39$; unpaired t-test).

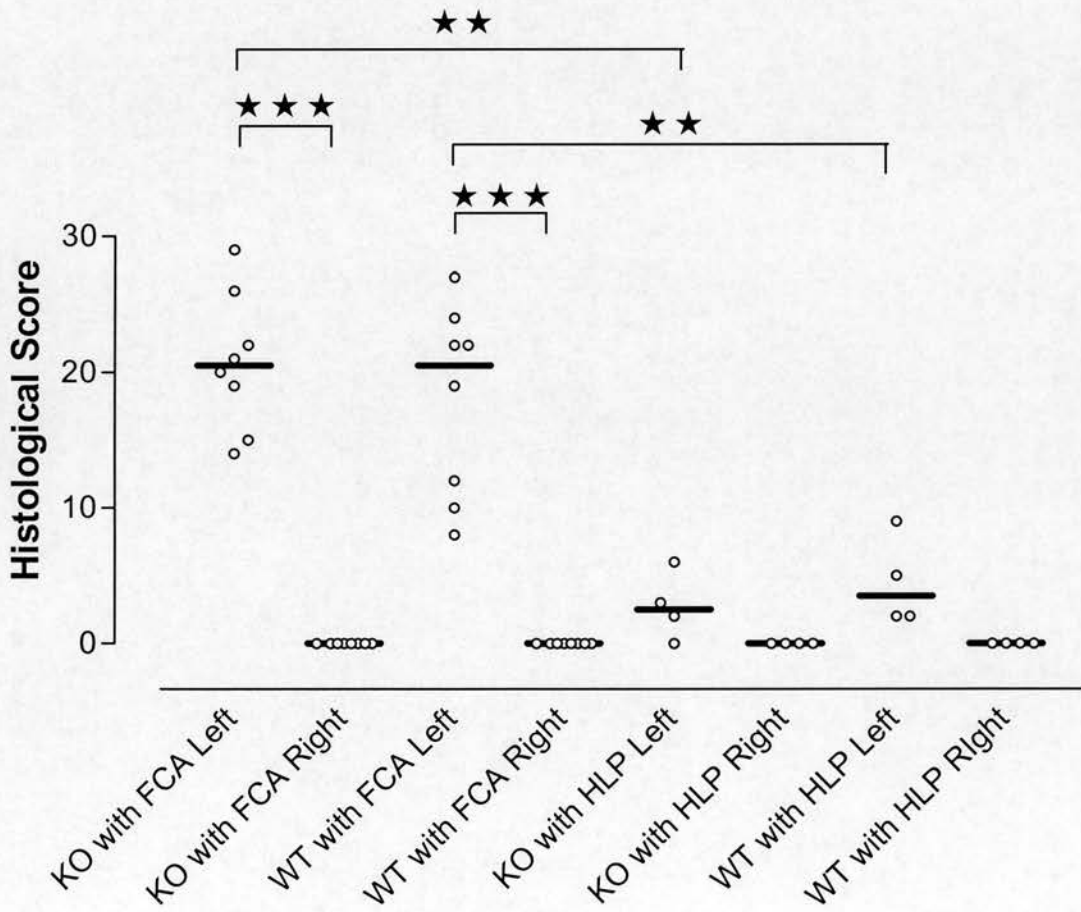


Figure 6.40 – Activation of C-fibre polymodal nociceptors in the C57BL6 mouse knee joint

Multi-fibre neural discharge (left panel) recorded from the saphenous nerve showing the response evoked from individual fibres (right panel) by A) mechanical probing of the joint (at arrow) using a small diameter plastic probe (activated fibres 1,2, and 3) and B) the close-arterial injection (at arrow) of capsaicin (330 pmol, which activated fibres 1, 2, and 3). Filters were set to allow only discharge above noise level to be captured. Some of the individual fibres that comprise the response are shown on the right panel.

The fibres were thought to be C-fibre polymodal nociceptors since they responded to noxious mechanical pressure on the joint and were also activated by capsaicin.

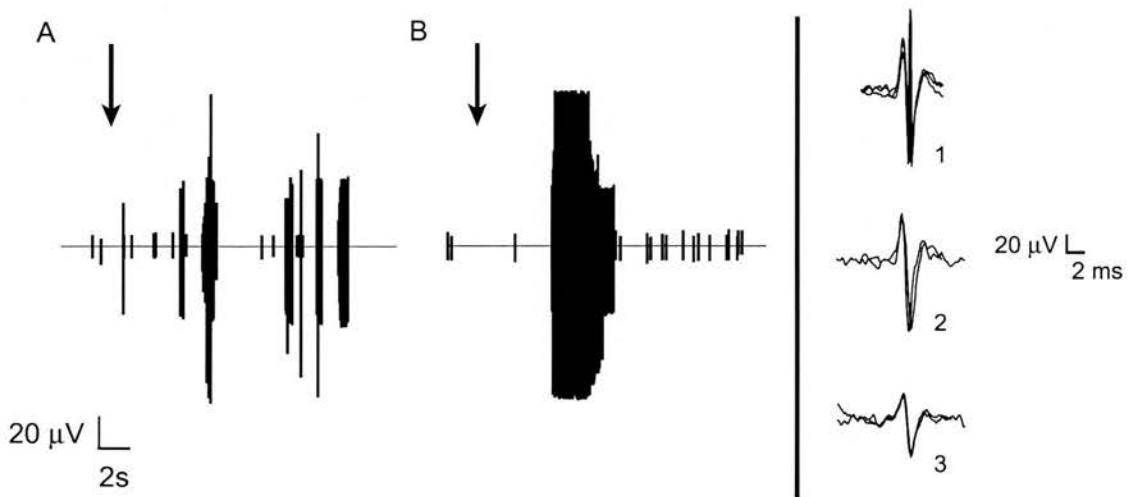


Figure 6.41 – Capsaicin activates sensory afferents innervating the C57BL6 mouse knee joint in a dose- dependent manner

Typical log dose response curves displaying the dose-related increase in discharge frequency (impulses s⁻¹) and the total number of action potentials (impulses) evoked by close arterial injection of capsaicin (33 – 980 pmol) in a normal mouse knee joint. A,B, and C represent three different animals.

Capsaicin evoked action potential discharge from nociceptive afferents contained in the saphenous nerve and innervating the normal mouse knee joint in a dose-dependent manner.

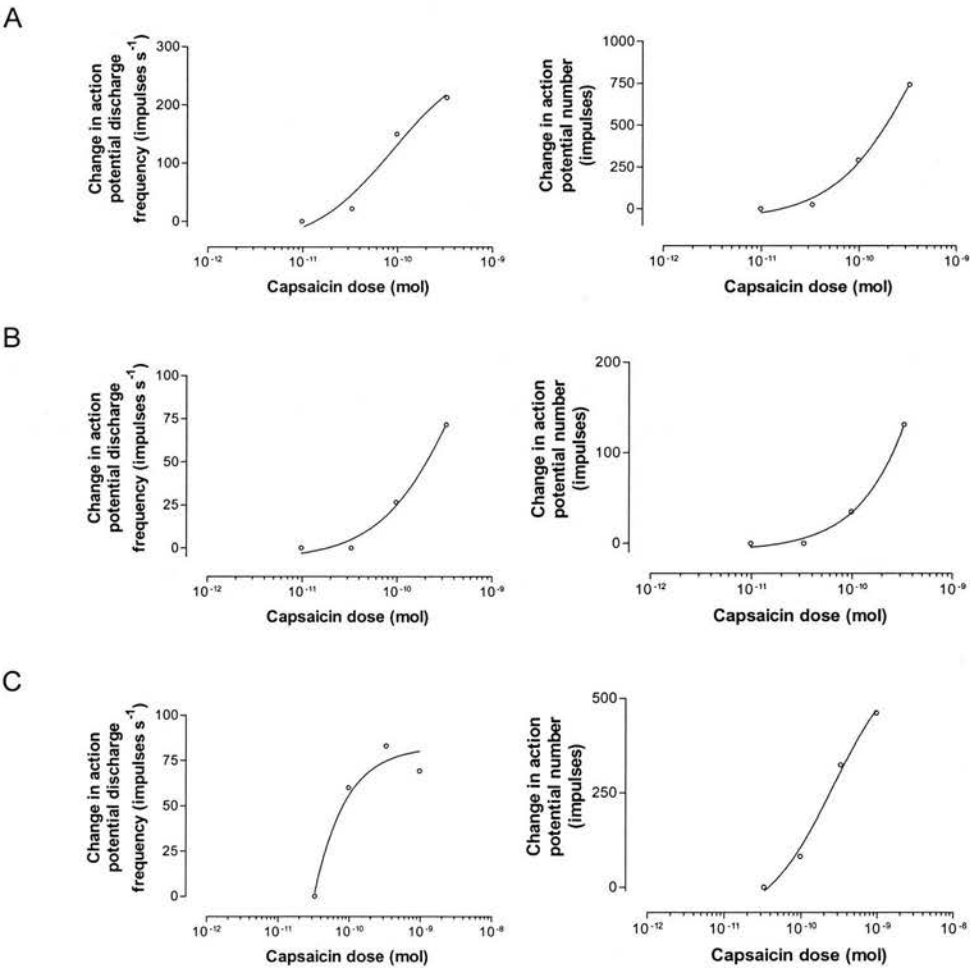


Figure 6.42 – Bradykinin activates sensory afferents innervating the C57BL6 mouse knee joint in a dose- dependent manner

Typical log dose response curves displaying the dose-related increase in A) discharge frequency (impulses s^{-1}) and B) the total number of action potentials (impulses) evoked by close arterial injection of bradykinin (94 – 2830 pmol) in a normal mouse knee joint.

Bradykinin evoked action potential discharge from nociceptive afferents contained in the saphenous nerve and innervating the normal mouse knee joint in a dose-dependent manner.

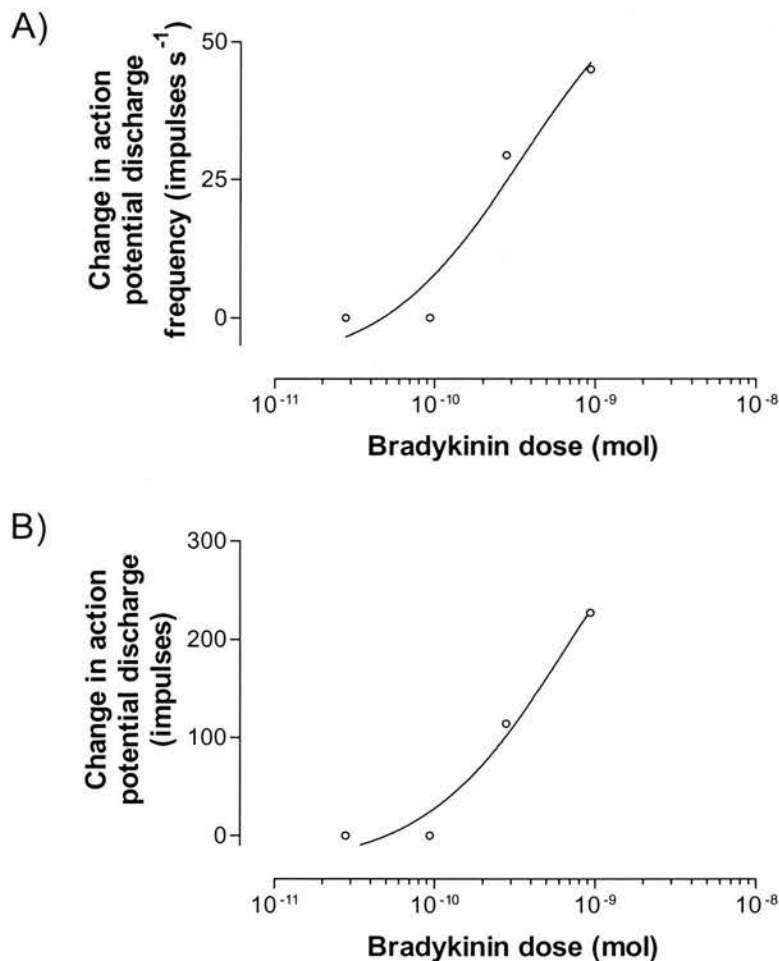
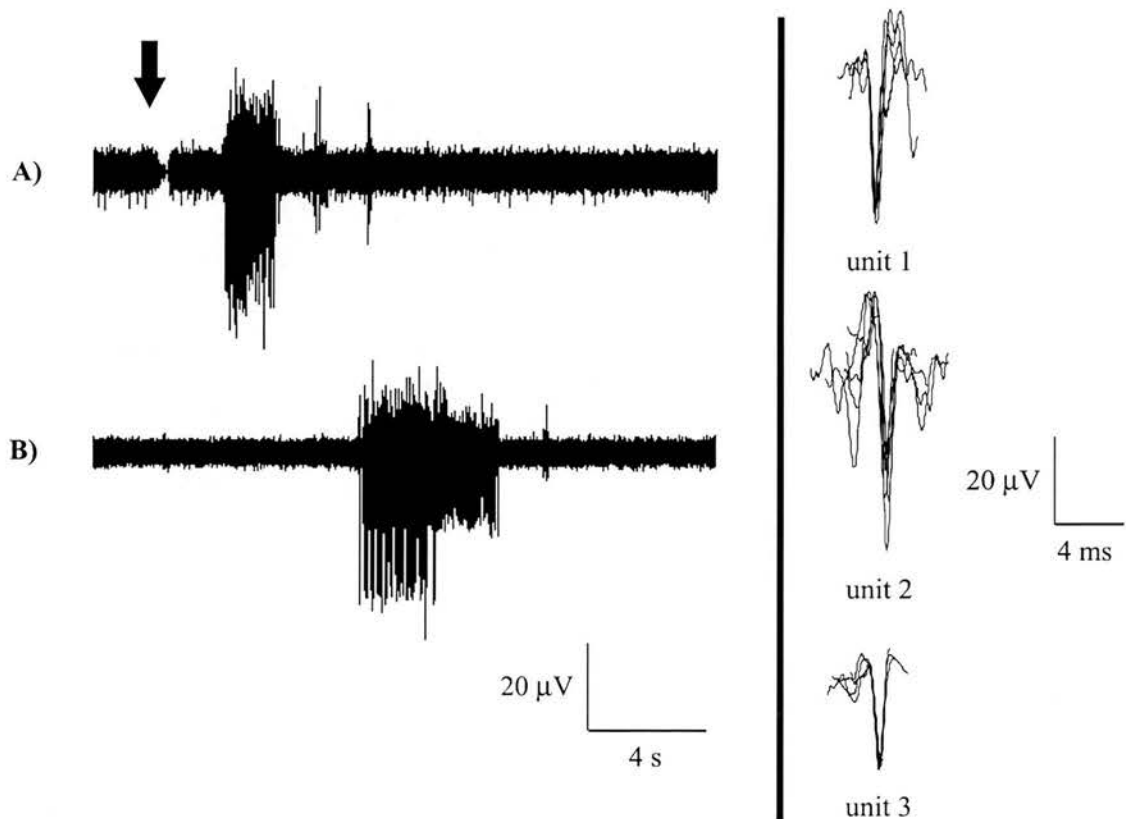


Figure 6.43 – Typical responses to capsaicin and bradykinin in afferents of the MAN in the C57BL6 mouse knee joint

Multi-fibre neural discharge (left panel) recorded from the saphenous nerve showing the response evoked by close-arterial injection (at arrow) of A) capsaicin (330 pmol, which activated fibres 1, 2, and 3) and B) bradykinin (940 nmol, activated fibres 1 and 2). Data in the left panel is the raw signal shown without the removal of the noise band. Individual fibres that comprise the response are shown on the right panel.

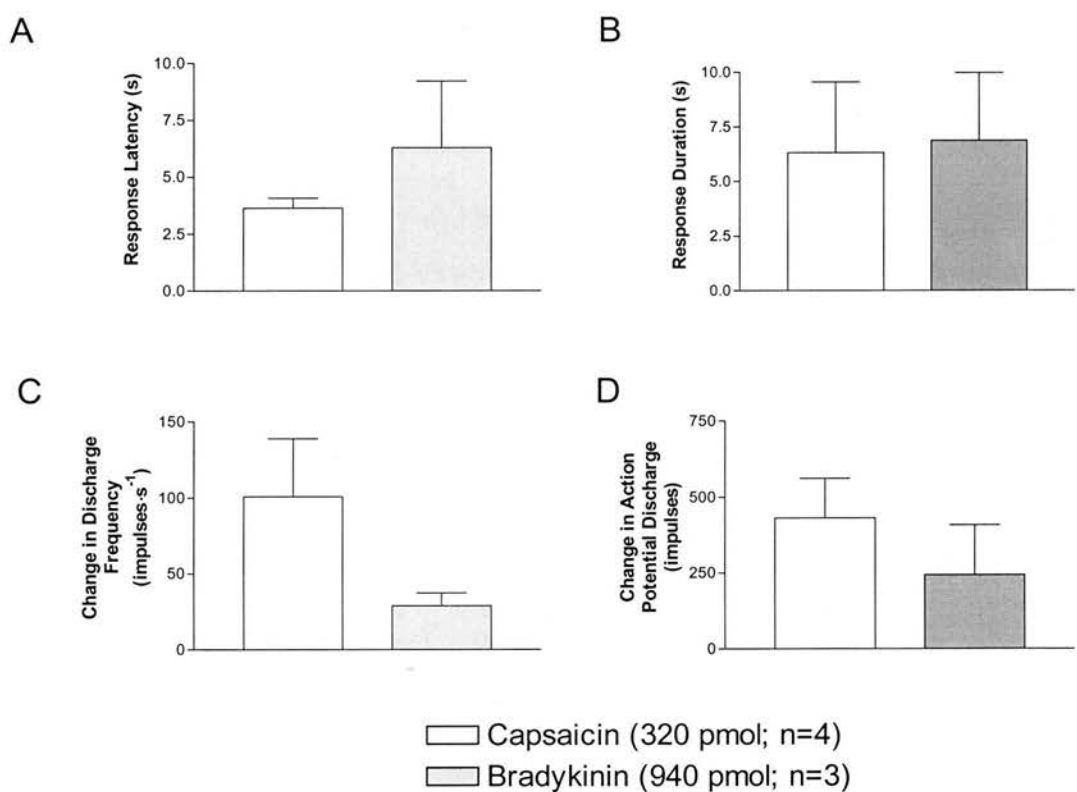


It is possible to record excitation in afferents that innervate the mouse knee joint by the close arterial injection of capsaicin and bradykinin.

Figure 6.44 – Comparison of the excitation of mouse knee joint afferents evoked by capsaicin and bradykinin

A) Latency to onset, B) duration of the response, C) action potential discharge frequency and D) total number of action potentials elicited following a close arterial injection of capsaicin (330 pmol) or bradykinin (940 pmol). Data shown are mean \pm sem for pooled responses.

Capsaicin had a more rapid onset and a greater discharge frequency when compared to bradykinin.



7. General Discussion and Future Directions

7.1 PROPERTIES OF NOCICEPTORS INNERVATING NORMAL AND ARTHRITIC RAT KNEE JOINT

One of the primary aims of this thesis was to examine peripheral nociceptors that innervate knee joints in the rat to determine whether or not there are differences in their response characteristics in normal and arthritic (chronic inflammation) states. In the rat a higher proportion of afferents exhibited spontaneous activity in arthritic joints when compared to normal joints. Guilbaud *et al* (1985) showed that, in addition to an increase in the proportion of afferents showing spontaneous activity, the afferents that innervate the arthritic rat knee joint also discharged at a higher frequency. This was confirmed in this lab in the rat ankle joint (Asghar, 1995). However, results presented in this thesis did not provide evidence for this, as there was no significant difference in the frequency of the spontaneous excitation of afferents innervating normal and arthritic knee joints.

In addition to investigating spontaneous activity the excitation evoked by a number of chemical mediators was examined in afferents innervating normal and arthritic knee joints. Afferents excited by capsaicin (VR₁ receptor agonist) and $\alpha\beta$ meATP (P2X₁, P2X₃ and P2X_{2/3} receptor agonist) showed no sensitisation to activation in the arthritic joint. It has been reported that both the VR₁ and P2X₃ receptors become sensitised during peripheral acute inflammation (Carlton & Coggeshall, 2001; Xu & Huang, 2002). Sensitisation of peripheral nociceptors mediated by these receptors appears to be the result of an increase in the receptor population within 48 hours of the induced inflammation. The acute nature of the inflammation means that these results may not be relevant to a chronic inflammatory

condition. During chronic inflammation induced by the repeated intra-articular injection of FCA there was conflicting evidence as to the sensitivity of VR₁ receptors. Results using capsaicin as a ligand at VR₁ show there is desensitisation of the afferent excitation in the chronically inflamed joint, whereas with anandamide, also known to be a ligand for VR₁ receptors (Olah *et al.*, 2001; Smart *et al.*, 2000; Zygmunt *et al.*, 1999), there was no desensitisation. In the FCA-induced arthritis model excitatory responses to both capsaicin and $\alpha\beta$ meATP are decreased as the receptors are rapidly desensitised by repeated stimulation. During chronic inflammation when the levels of endogenous ligand are elevated the receptors may become desensitised over time. If the sensitivity of the VR₁ receptor decreased during chronic inflammation, then one would predict that a corresponding increase in the threshold to thermal nociception would occur. There are many reports in the literature referring to the induction of thermal hyperalgesia in cutaneous and joint afferents following acute inflammation, but there are no reports in which the thermal properties of nociceptors have been investigated in chronically inflamed tissue. In one human study that examined thermal sensitivity in the thumb joint of patients with OA there was hyperalgesia to an applied thermal stimulus (Farrell *et al.*, 2000). However, as the stimulus was applied over the joint and the relative contribution of skin and joint nociceptors in the overall pain response are currently not known it is difficult to draw conclusions about the thermal sensitivity of joint nociceptors specifically.

A further finding of this thesis is that the endogenous cannabinoid, anandamide, was able to excite peripheral nociceptors *in vivo* through activation of the VR₁ receptor. Since the capsaicin-evoked response was inhibited in the

chronically inflamed joint, the anandamide-mediated excitation was examined in nociceptive afferents innervating the normal and the arthritic knee joint. It transpired that excitation by anandamide of afferents innervating arthritic joints did not differ significantly from that observed in normal joints. Although the response to anandamide could be blocked with the VR₁ receptor specific antagonist, capsazepine, there appeared to be no desensitisation of the VR₁ receptor during chronic inflammation. Additionally, repeated injections of anandamide, even at a maximal dose (2900 nmol), did not desensitise the activation of afferents in either normal or arthritic joints.

Although anandamide is a full agonist at the human VR₁ receptor (Smart *et al.*, 2000), it is much less potent than capsaicin and other vanilloid analogs (Ralevic *et al.*, 2001). Experiments with anandamide were limited to a maximal concentration (2900 nmol) due to the limited solubility of the molecule and potentially the level of excitation at the VR₁ receptor in our model was sufficient to produce activation but not desensitisation. It is possible that anandamide binds to a different site on the VR₁ receptor or activates the channel through a different mechanism. Activation of the receptor complex through a different mechanism could account for the fact that, in contrast to capsaicin, anandamide is non-pungent. However, the actions of anandamide and capsaicin are both blocked by the competitive antagonist capsazepine so it is unlikely that there is a secondary binding site. The fact that anandamide is non-pungent is not linked to its lower potency at VR₁ receptors because the capsaicin analog, olvanil, has been shown to be tenfold more potent at the human VR₁ receptor - while still remaining non-pungent (Ralevic *et al.*, 2001). It has been suggested that olvanil is non-pungent because the activation kinetics of the

ligand-receptor interaction are slower than the rate at which it inhibits the generation of action potentials through desensitisation (Liu *et al.*, 1997).

The two ligand-gated ion channels detailed above did not show any evidence of sensitisation during chronic inflammation. In contrast, bradykinin, which acts through a G-protein coupled receptor (GPCR), evoked increased afferent discharge from afferents innervating arthritic joints. As discussed in Chapter 3 expression of the B1 receptor is upregulated during inflammation, and the increased afferent discharge could be the result of combined excitation of B1 and B2 receptors on peripheral terminals.

FUTURE DIRECTIONS

The sensitisation of peripheral nociceptors during chronic inflammation is an area of research that requires much more investigation. The role of the large number of mediators released during chronic inflammation in the excitation and/or inhibition of peripheral nociceptors is for the most part unknown. The information that is available concerning the chemical excitation of joint afferents is extrapolated from experiments on cutaneous nociceptors. Detailed characterisation of joint nociceptors is necessary to determine whether they have a unique pharmacological profile, or whether the pharmacological characteristics of nociceptors are uniform and independent of the tissue they innervate.

Mediators that need to be investigated are the various prostaglandins and thromboxanes and an ever-increasing list of cytokines, all of which play a critical role in cell-cell interactions during inflammation and as such may also influence sensory neurons.

A number of questions arise from the work in this thesis, including the mechanism for desensitisation of the VR₁ receptor during chronic inflammation, that cannot be answered using whole animal electrophysiology and instead would be better addressed by in vitro methods. However further studies using this model should enable the role of putative mediators and their pharmacological receptors in the activation of nociceptors to be examined. To continue previous work in this laboratory exploring the activation of nociceptors by bradykinin (Kelly, 1998; Kelly *et al.*, 1995a; Kelly *et al.*, 1995b) it should be possible to use selective antagonists to determine the role of the B1 receptor in the increased nociceptive discharge evoked from afferents innervating the arthritic knee joint. Furthermore, recent reports have shown that the population of P2X receptors present on sensory nerves and peripheral terminals is more heterogeneous than previously thought, as it includes P2X₁, P2X₂ and various combinations of P2X₂ and P2X₃ subunits to produce homomer and heteromer channels (Liu *et al.*, 2001; Pankratov Yu *et al.*, 2001; Petruska *et al.*, 2000). To determine which P2X receptor is being activated on peripheral nociceptors will require the development of subtype specific agonists or antagonist, or the generation of subtype specific P2X receptor knockout animals. Until these pharmacological tools become available the P2X subtype involved in nociceptor transmission and how it behaves during chronic inflammation cannot be determined.

7.2 MODULATION OF NOCICEPTORS BY INFLAMMATORY MEDIATORS

Another of the aims was to examine whether or not the neuropeptide somatostatin could modulate spontaneous and/or chemically evoked stimulation of

sensory nociceptors. Although there is an extensive literature on the use of somatostatin as an anti-inflammatory and analgesic (see Chapter 4), in the FCA-induced model of mild chronic inflammation there was no effect on nociceptors innervating the knee joint. Somatostatin did not inhibit spontaneous discharge in normal or arthritic joints, nor did it alter the excitation evoked by chemical stimuli - capsaicin, bradykinin, or $\alpha\beta$ meATP. It is difficult to know why there was no effect; somatostatin is released from sensory terminals innervating the skin of the rat hind paw in response to noxious stimulation and it has been shown that both locally released and exogenously applied somatostatin can inhibit noxious mechanical stimulation of these afferent C-fibres (Carlton *et al.*, 2001a; Carlton *et al.*, 2001b).

The endogenous cannabinoid anandamide is also released from sensory neurons during inflammation. Anandamide can act via CB₁ receptors, and as shown in Chapter 5, can also act on the VR₁ receptor. Both of these receptors play a role in nociceptive processing. However, anandamide did not inhibit chemically evoked excitation of sensory afferents innervating either normal or arthritic rat knee joints caused by capsaicin, bradykinin or $\alpha\beta$ meATP.

Questions that arise from these studies concern the fact that both somatostatin and anandamide compounds have been shown to be analgesic, and yet they were unable to inhibit activation of peripheral nociceptor terminals by chemical stimuli. From this one could conclude that they act centrally or on the cell bodies in the DRG rather than the peripheral nociceptive terminals. Alternatively, it could be an inherent fault of the way in which the drug is administered. Somatostatin acts as an anti-inflammatory through its inhibition of the action of substance P and it may be that levels of somatostatin have to be increased for a longer period to cause

analgesia. Thus the effects of a single bolus injection of the drug may be too transient to inhibit transmission.

FUTURE DIRECTIONS

To address the concern that somatostatin or anandamide are not given sufficient time to act, further experiments could be done where the drugs are administered over a longer period either using an infusion pump or by repeated injections. This would allow a high systemic level of the drug to be maintained. Furthermore, animals with FCA-induced unilateral arthritis receiving either anandamide or somatostatin have not been tested in the weight-bearing paradigm to determine their effectiveness in inhibiting the pain associated with chronic inflammation. Once it has been established that these compounds can effectively modulate nociception on a behavioural level, then the mechanisms by which this is occurring should be investigated electrophysiologically.

Recent interest in the analgesic and anti-inflammatory properties of endogenous and exogenous cannabinoids has opened up a whole new range of compounds to investigate in FCA-induced model of chronic inflammation. Further experiments could be performed to determine the efficacy of the other endogenous cannabinoid receptor ligand, 2-arachidonyl glycerol, in modulating chemical activation of peripheral terminals. Additionally, there are a number of CB₁ and CB₂ receptor agonists and antagonists available, which could be used to characterise these receptors and to establish their role in the activation and sensitisation of peripheral nociceptors during chronic inflammation.

7.3 MURINE UNILATERAL ARTHRITIS AND ELECTROPHYSIOLOGICAL RECORDING FROM AFFERENTS INNERVATING THE MOUSE KNEE JOINT

Part of this thesis was dedicated to the development of a novel model of murine arthritis and establishing whether it is feasible to record activity from sensory afferents innervating the mouse knee joint.

FCA-INDUCED UNILATERAL ARTHRITIS IN THE MOUSE KNEE JOINT

In the development of a new animal model of arthritis it is important to consider two questions: does the model mimic the pathology of the clinical condition and can it predict effective therapeutic treatments in man. Results show that repeated intra-articular injections of FCA induced chronic joint inflammation. Histology demonstrated that the inflammation has a number of the characteristics of arthritis, including pannus formation, bone and cartilage destruction, and infiltration of a large number of inflammatory cells, oedema, and fibrous deposits through out the tissue. Additionally, contrary to other models of arthritis in the mouse (see Chapter 6), this model appears to be consistent across two mouse strains. This allows comparisons to be drawn on differences between strains – particularly important when studying transgenic mice, which vary widely in terms of their genetic background.

Although no experimental model perfectly mimics the human disease, animal models of arthritis (both RA and OA) have been very effective in the prediction of therapeutic effectiveness of drugs in man. Drugs that inhibit the progression of experimental arthritis, such as steroids, NSAIDS, COX-2 inhibitors, and pro-inflammatory cytokine blockers, are routinely used in the treatment of human

arthritic conditions (Bendele *et al.*, 1999; van den Berg, 2001a; van den Berg, 2001b; van den Berg, 2000b). FCA-induced unilateral arthritis in the mouse has shown similar properties, in that inflammation of the joint is inhibited by treatment with steroid (e.g. prednisolone) and NSAID (e.g. indomethacin) drugs.

An additional consideration in designing models for disease is the welfare of experimental animals. Systemic models of chronic inflammation produce a very severe polyarthritis that is stressful and causes severe suffering in the animal. The unilateral and mild nature of the FCA-induced arthritis means that the inflammation is isolated to a single joint, thereby minimising the discomfort and distress to the mouse. Furthermore, a unilateral inflammation provides an intra-animal control allowing comparisons to be made between an injected and uninjected joint. Finally, in the rat, the level of inflammation induced by the injection of FCA is dose dependent. Therefore, an appropriate dose can be given to induce a mild-moderate joint inflammation, sufficient for the examination of the condition without causing severe discomfort in the animal. It remains to be investigated whether FCA-induced inflammation in the mouse is similarly dose-dependent, but this can easily be established by further experiments.

Future directions

There are a number of issues that still need to be resolved in order to validate the model as a good predictor of treatments for arthritis. Experiments need to be done that examine the role of IL-1 β and TNF α in the initiation and maintenance of chronic joint inflammation. In addition, in order to be used as true measures of effective treatment, steroids and NSAIDS should be administered orally – these

experiments would not take much time but would further confirm the effectiveness of the model.

The development of apparatus for the measurement of weight bearing on the hind limbs is required to facilitate the objective measurement of the hyperalgesia associated with inflammation of joints in mice. Similarly, a force transducer linked to a pair of forceps could provide an objective measurement of the withdrawal latency of a limb to (quantified) applied pressure.

Once the model is fully characterised it can be used as an effective tool for the examination of transgenic mouse lines in which genes that are considered to be of particular relevance to chronic inflammation or peripheral nociception have been modified. To gain the most information from this system it would be best to examine transgenics where the mutation can be temporally regulated using chemically inducible promoters. Additionally, using site-specific promoters it may be possible to restrict the mutation to a particular tissue (such as peripheral nerves).

ELECTROPHYSIOLOGICAL RECORDING FROM MOUSE KNEE JOINT AFFERENTS IN VIVO

Results in this thesis detail the development of a method for recording from peripheral afferents innervating the knee joint in a live, anaesthetised mouse. There is no mention in the literature of other groups having achieved this. This method can be used to record mechanical and chemical stimulation of peripheral nociceptors.

Future directions

As much of the work presented in this thesis is preliminary, based on time-consuming and technically demanding developmental experiments, there is scope for

a considerable amount of work to be performed in this area. Very little is known about the innervation of the mouse knee joint, and it is only recently that the types of fibres present in the mouse MAN were examined using electron microscopy (Ebinger *et al.*, 2001). As so little is known about the types of fibres that innervate the mouse knee, there is virtually no information on the pharmacological profile of these afferents. Future experiments in this area could examine a whole range of targets and would provide insight into the types of receptors that are present on mouse peripheral afferents and how this compares with the situation in other species.

Probably the most interesting use of neural recordings from afferents in the mouse knee joint would be combining it with existing technology for the production of transgenic mice. A large number of transgenic mouse lines with genetic alterations relevant to nociception already exist. Using transgenic animals that over express a particular gene or have it completely removed, one could quickly determine whether the activation and/or sensitisation of peripheral nociceptors were due to a particular receptor or receptor subtype. If this was combined with a measurement of weight bearing or hyperalgesia to pressure applied to the knee joint, it should be possible to correlate genetic alterations directly with changes in nociceptor activation and ultimately the perception of pain.

7.4 GENERAL CONCLUSIONS

The results presented in this thesis contribute to our overall knowledge concerning the pharmacological profile of nociceptive afferents innervating the knee joint in rats and mice. They showed that the reported anti-nociceptive effect of somatostatin is not mediated by action on peripheral nociceptors or the inhibition of

tested algogens. Furthermore anandamide is able to directly activate sensory afferents via VR₁ receptors.

In addition, a new model of chronic unilateral arthritis – based on repeated intra-articular injections of FCA – has been established. Using this model, and P2X₇ knockout mice, it was demonstrated that P2X₇ purinoceptors do not play a role in the induction of inflammation and hyperalgesia in unilateral arthritis. Finally, a new technique was developed for direct measurement of mechanically and chemically evoked discharge from the peripheral terminals of afferent nociceptors innervating the mouse knee joint.

Results of studies on the peripheral terminals of sensory afferents provide information relevant to the design and testing of therapeutic targets for the modulation of nociception. Selective peripheral analgesics targeting nociceptive terminals would minimise unwanted actions of compounds within the central nervous system. Determining how the properties of nociceptors (and their associated drug receptors) change during chronic inflammation will provide valuable insight into molecular and cellular changes that are responsible for the sensitisation and activation of peripheral nociceptor during chronic inflammatory conditions. This information will be vital for the design and development of better analgesic and anti-inflammatory drugs.

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Index of Figures and Tables

Figure 2.1 – Experimental set-up for neuronal recording from rat MAN	56
Figure 2.2 – Schematic of signal processing of neural recording	1
Figure 3.1 – Proportion of afferent nerves showing spontaneous firing in recordings from normal and arthritic rat knee joints	77
Figure 3.2 – Basal spontaneous activity in fibres of the MAN in normal and arthritic rat knee joints	78
Figure 3.3 – Typical responses evoked by capsaicin, bradykinin and ATP in a multi- unit recording from the MAN in a normal rat knee joint	79
Figure 4.1 – Structure of somatostatin and synthetic analog octreotide	85
Figure 4.2 – Effect of octreotide on spontaneous discharge in afferents innervating normal and arthritic rat knee joints	98
Figure 4.3 – Evoked responses to capsaicin, bradykinin, and $\alpha\beta$ meATP following octreotide in normal rat knee joints	99
Figure 4.4 – Blood pressure response evoked by octreotide and somatostatin-14 in a normal rat	100
Table 5.1 – Effect of anandamide on evoked excitation from nociceptive afferents by ATP, bradykinin and capsaicin	116
Table 5.2 – Responses evoked by bradykinin and $\alpha\beta$ meATP before and after capsazepine	117
Figure 5.1 – Anandamide excites a population of sensory afferents in the normal rat knee joint.	118
Figure 5.2 – Proportion of C and A δ fibres activated by anandamide	119
Figure 5.3 – Anandamide activates sensory afferents in a dose- dependent manner	120
Figure 5.4 – Excitatory effects of anandamide on afferent discharge recorded from normal and arthritic knee joints	121
Figure 5.5 – Comparison of the excitatory response recorded from nociceptive afferents in normal and arthritic rat knee joints following injection of anandamide	122
Figure 5.6 – Comparison of the excitation evoked by capsaicin and anandamide from articular nociceptive afferents innervating normal rat knee joints.	123
Figure 5.7 – Effect of capsazepine on afferent discharge evoked by capsaicin and anandamide from nociceptive afferents innervating a normal rat knee joint. ..	124

Table 6.2 - Explanation of pain scores for hyperalgesia from a joint squeeze.....	182
Figure 6.1 – Schematic of the experimental set up for recording from saphenous nerve in mice	183
Figure 6.2 – Effect on joint diameter of a single sub-cutaneous injection of FCA in the ankle of DBA1 mice.....	184
Figure 6.3 – Effect on joint diameter of a single sub-cutaneous injection of FCA (or a single plus a booster injection) in the knee of DBA/1 mice.....	185
Figure 6.4 – Effect on knee diameter of mBSA-induced inflammation in the knee of C57BL6 mice.....	186
Figure 6.6 – Effect on joint diameter of repeated i.art. injections of FCA into the knee of DBA/1 mice.	188
Figure 6.7 – Schematic of inflammatory response to repeated intra-articular injections of FCA	189
Figure 6.8 – Effect of repeated injections of FCA on body weight in DBA/1 mice	190
Figure 6.9 – Effect of repeated injections of FCA on mobility in DBA/1 mice	191
Figure 6.10 – Effect of FCA on pain evoked by noxious pressure applied to the knee joint in DBA/1 mice	191
Figure 6.10 – Effect of FCA on pain evoked by noxious pressure applied to the knee joint in DBA/1 mice	192
Figure 6.11 – Histopathology of DBA/1 mice receiving repeated intra-articular injections of FCA	193
Figure 6.12 – Histopathology of DBA/1 mice receiving repeated intra-articular injections of FCA	194
Figure 6.13 – Effect on joint diameter of repeated injections of FCA in the knee of C57BL6 mice.....	195
Figure 6.14 – Effect of repeated injections of FCA on body weight in C57BL6 mice	196
Figure 6.15 - Effect of repeated injections of FCA on mobility in C57BL6 mice...	197
Figure 6.16 – Effect of FCA on pain evoked by noxious pressure applied to the knee joint in C57BL6 mice	198
Figure 6.17 – Histopathology of DBA/1 mice receiving repeated intra-articular injections of FCA	199
Figure 6.18 – Histopathology of C57BL6 mice receiving repeated intra-articular injections of FCA	200

Figure 6.19 – Comparison of knee diameter during FCA-induced unilateral arthritis in DBA/1 and C57BL6 mice	201
Figure 6.20 – Comparison of mobility during FCA-induced unilateral arthritis in DBA/1 and C57BL6 mice	202
Figure 6.21 – Comparison of pain evoked by noxious pressure applied to the knee joint in DBA/1 and C57BL6 mice.....	203
Figure 6.22 – Comparison of histological scores in DBA/1 and C57BL6 mice receiving repeated intra-articular injections of FCA	204
Figure 6.23 – Effect of prednisolone on knee diameter during established FCA induced unilateral arthritis in DBA/1 mice	205
Figure 6.24 – Effect of prednisolone on body weight during established FCA-induced unilateral arthritis in DBA/1 mice	206
Figure 6.25 – Effect of prednisolone on the mobility during established FCA-induced unilateral arthritis in DBA/1 mice	207
Figure 6.26 – Effect of prednisolone on pain evoked by noxious pressure during established FCA-induced unilateral arthritis in DBA/1 mice.....	207
Figure 6.26 – Effect of prednisolone on pain evoked by noxious pressure during established FCA-induced unilateral arthritis in DBA/1 mice.....	208
Figure 6.27 – Effect of prednisolone on inflammation and joint destruction during established FCA-induced unilateral arthritis in DBA/1 mice.....	209
Figure 6.28 – Effect of prednisolone on the histological score during established FCA-induced unilateral arthritis in DBA/1 mice	210
Figure 6.29 – Effect of indomethacin on knee diameter during established FCA-induced unilateral arthritis in C57BL6 mice	211
Figure 6.30 – Effect of indomethacin on body weight during established FCA-induced unilateral arthritis in C57BL6 mice.....	212
Figure 6.31 – Effect of indomethacin on mobility during established FCA-induced unilateral arthritis in C57BL6 mice.....	213
Figure 6.32 – Effect of indomethacin on pain evoked by noxious pressure during established FCA-induced unilateral arthritis in C57BL6 mice	213
Figure 6.32 – Effect of indomethacin on pain evoked by noxious pressure during established FCA-induced unilateral arthritis in C57BL6 mice	214
Figure 6.33 – Effect of indomethacin on inflammation and joint destruction in established FCA-induced unilateral arthritis in C57BL6 mice.	215

Figure 6.34 – Effect of indomethacin on the histological score in established FCA-induced unilateral arthritis in C57BL6 mice	216
Figure 6.35 – Effect of FCA-induced unilateral arthritis on knee diameter in P2X7 knock out mice.	217
Figure 6.36 – Effect of FCA on body weight in P2X7 KO and WT mice.	218
Figure 6.37 – Effect of repeated injections of FCA on mobility in P2X7 KO and WT mice	219
Figure 6.38 - Effect of repeated injections of FCA on pain evoked by noxious pressure in P2X7 KO and WT mice	220
Figure 6.39 - Effect of FCA on the histological score in P2X7 KO and WT mice..	221
Figure 6.40 – Activation of C-fibre polymodal nociceptors in the C57BL6 mouse knee joint	222
Figure 6.41 – Capsaicin activates sensory afferents innervating the C57BL6 mouse knee joint in a dose- dependent manner	223
Figure 6.42 – Bradykinin activates sensory afferents innervating the C57BL6 mouse knee joint in a dose- dependent manner	224
Figure 6.43 – Typical responses to capsaicin and bradykinin in afferents of the MAN in the C57BL6 mouse knee joint	225
Figure 6.44 – Comparison of the excitation of mouse knee joint afferents evoked by capsaicin and bradykinin	226

Appendix I - Drugs and Solutions

Drugs used in this study

Generic Name	Chemical Name	Formula Weight (g mol ⁻¹)	Supplier	Solvent
ATP	adenosine 5' triphosphate disodium salt	551.1	Sigma	PBS
$\alpha\beta$ meATP	α,β -methylene adenosine triphosphate lithium salt	505.2 (free acid)	Sigma	PBS
Anandamide	N-(2-hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide	347.5	Tocris	Soya oil: water (1:4), PBS
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	1060.2	Sigma	PBS
Capsaicin	8-methyl-N-vanillyl-6-nonenamide	305.4	Sigma	10% Tween 80, 10% ethanol, PBS
Capsazepine	N-[2-(4-Chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide	376.9	Sigma	10% Tween 80, 10% ethanol, PBS
Octreotide	D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr-ol	1019.3		PBS
Somatostatin-14	Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys	1637.9	Sigma	PBS

Solutions

Saline: 0.9 g NaCl in 100 ml distilled water

Phosphate Buffered Saline: 40.5 ml 0.2M Na₂HPO₄.12H₂O in saline
(PBS) 9.5 ml 0.2M Na₂H₂PO₄.2H₂O in saline
50 ml saline

10% Formalin: 10 ml 40% formaldehyde (w/v)
0.9 g NaCl
90 ml distilled water

Appendix II - Publications



SPECIAL REPORT

Anandamide activates peripheral nociceptors in normal and arthritic rat knee joints

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The effects of the endogenous cannabinoid anandamide were studied on peripheral, polymodal nociceptors recorded from normal and chronically inflamed (Freund's adjuvant) knee joint afferents in rats anaesthetized with pentobarbitone. Anandamide (860 nmol) caused a rapid, short lasting excitation of a sub-population of capsaicin-sensitive nociceptive afferents in normal knee joints (7.2 ± 2.3 impulses s^{-1} ; $n=15$ units from five animals). In arthritic joints there were 9.7 ± 3.0 impulses s^{-1} ($n=11$ from six animals), which was not significantly different from normal joints. The excitation was dose dependent (8.6 – 2900 nmol) and mediated by activation of the vanilloid receptor (VR₁) as it was abolished by the VR₁ antagonist capsazepine (1 mg kg^{-1}). Our results show that anandamide, at high doses, can activate nociceptive afferents innervating the rat knee joints, in contrast with its widely described analgesic actions.

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Keywords: Sensory nerves; anandamide; adjuvant arthritis; knee joint; afferent nociceptors; vanilloid receptor

Abbreviations: $\alpha\beta$ meATP, α,β -methyleneadenosine 5'-triphosphate; anandamide, arachidonyl ethanolamide; ATP, adenosine triphosphate; capsaicin, 8-methyl-N-vanillyl-6-nonenamide; CGRP, calcitonin-gene-related peptide; PBS, phosphate buffered saline; VR₁, vanilloid receptor

Introduction Following the discovery of anandamide as an endogenous ligand for the CB (cannabinoid) receptor, there has been intense interest in its physiological function. An important putative role for anandamide is as a modulator of nociception, in part involving activation of the CB₁ receptors on sensory neurones and the subsequent inhibition of the release of CGRP (Richardson *et al.*, 1998). Reports have also linked anandamide with the recently cloned vanilloid receptor subtype 1 (VR₁) which is sensitive to capsaicin (Caterina *et al.*, 1997). The action of anandamide on the VR₁ receptor has been demonstrated using transfected human receptors (Smart *et al.*, 2000), on sensory nerves controlling vasodilatation in isolated arteries (Zygmunt *et al.*, 1999), and the amplitude of electrically-evoked contractions in the mouse isolated vas deferens (Ross *et al.*, 2001). The VR₁ agonist capsaicin is a potent activator of C-fibre polymodal nociceptors in the periphery. In view of the reported actions of anandamide, and its structural relationship to capsaicin and other vanilloid agonists, we hypothesized that anandamide can directly activate capsaicin nociceptors and have investigated those innervating the rat knee joint. Using a unilateral adjuvant-induced arthritis model in the rat (Dowd *et al.*, 1998) we have also investigated the effects of anandamide to determine whether the responsiveness of these joint nociceptors was altered during experimental inflammation.

Methods Experiments were performed on five normal and six arthritic male Wistar rats (body weight range 280–470 g; mean \pm s.e.mean 374 ± 19 g).

Induction of arthritis Freund's Complete Adjuvant (FCA, 0.15–0.20 ml of 1 mg ml^{-1} heat killed *Mycobacterium tuberculosis* in paraffin oil, Sigma) was introduced into the left knee (stifle) joint by intra-articular injection under transient halothane anaesthesia (3% in oxygen). The animals were used for electrophysiological recording 14–35 days post-injection. Animals displayed mild but persistent unilateral arthritis as characterized by a significant increase in the diameter of the injected joint (injected 10.6 ± 0.1 mm; uninjected 9.8 ± 0.1 mm; $n=6$, $P<0.05$, Wilcoxon).

Surgical procedures Animals were anaesthetized with an intra-peritoneal (i.p.) injection of pentobarbitone (60 mg kg^{-1}) and the trachea was cannulated. Anaesthesia was maintained with an intravenous (i.v.) infusion of pentobarbitone (0.4 – 0.5 mg kg^{-1} min^{-1}) via a cannula inserted in the right femoral vein and the right carotid artery was cannulated for monitoring blood pressure. An additional catheter was inserted into the right femoral artery with its tip positioned in the lower abdominal aorta to allow for close arterial injection of drugs to the left knee joint. Animals were killed at the end of the experiment by an overdose of anaesthetic.

Electrophysiology Extracellular recordings were performed on filaments of the medial articular nerve (MAN) innervating the left knee joint using previously described techniques (Dowd *et al.*, 1998). Briefly, the left leg was fixed to a support

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and an incision was made on the medial aspect of the limb and the skin secured to form a pouch that was filled with heavy liquid paraffin. Three branches of the medial articular nerve (MAN) were exposed at the point where they leave the saphenous nerve. The saphenous nerve was cut centrally to prevent interference from efferent activity. One branch of the MAN was dissected from surrounding tissue and electrical activity was recorded from filaments, typically containing 1–4 afferent fibres, using a Pt-Ir bipolar electrode connected to an amplifier. Neural activity was viewed on an oscilloscope, digitally recorded and analysed using a personal computer running Spike 2 software (CED, Cambridge). Drugs were administered by close intra-arterial injection (total volume: 0.1 ml drug + 0.2 ml wash), completed within 2 s. The minimum time interval between injections was 20 min to minimize the possibility of desensitization.

Data analysis Drug effects were determined by comparing the action potential discharge frequency or the absolute number of events immediately following injection with that in the 15 s period immediately prior to injection. Data are expressed as either the mean change in action potential frequency (impulses s^{-1}) or the change in the absolute number of action potentials evoked during the response (impulses). The maximum possible dose for anandamide was 2900 nmol, the concentration of the stock solution. Differences between means were analysed statistically using the Mann–Whitney test for unpaired data and the Wilcoxon matched-pairs test for paired data. The null-hypothesis was rejected at $P < 0.05$.

Drugs Anandamide and capsazepine were purchased from Tocris (U.K.) and capsaicin, bradykinin, adenosine 5'-triphosphate (ATP) and α,β -methyleneadenosine 5'-triphosphate ($\alpha\beta$ meATP) were purchased from Sigma (U.K.). Capsaicin was dissolved in Tween 80 (10% v v $^{-1}$), ethanol (10% v v $^{-1}$) and PBS. Capsazepine stock (10 mg ml $^{-1}$) was dissolved in 20% cremophor EL (Sigma, U.K.) in distilled water. All drugs were diluted in PBS for injection.

Results In normal animals ($n=5$) recordings were made from five afferent fibres consisting of 15 units with receptive fields in the knee joint. These units were identified as either C-fibre polymodal nociceptors (14/15, 93%) or A δ -mechanonociceptors (1/15, 7%) according to their conduction velocities, mechanosensitivity, and their response to capsaicin (9 nmol, 18.2 ± 5.4 impulses s^{-1}). Basal discharge from these afferents was 0.01 ± 0.01 impulses s^{-1} . Recordings were also made in arthritic animals ($n=6$) from a total of six mechanosensitive afferents consisting of 11 units, all of which were C-fibre polymodal nociceptors and responded to capsaicin (9 nmol i.a.: 11.6 ± 2.0 impulses s^{-1}). Basal discharge was 0.04 ± 0.02 impulses s^{-1} which was not significantly different ($P > 0.05$, Mann–Whitney) from the untreated group.

Responses to anandamide in normal and arthritic knee joints Anandamide (8.6–2900 nmol) evoked an increase in afferent discharge in 64% (9/14) of C-fibre polymodal nociceptors but not in the single A δ -mechanonociceptor recorded from normal knee joints. In arthritic joints

anandamide (8.6–2900 nmol) evoked a response in 72% (8/11) of C-fibre polymodal nociceptors. Injection of vehicle had no effect in these fibres (soya oil: water (1:4), -0.1 ± 0.1 impulses s^{-1} , $n=5$).

The excitation evoked by anandamide in the sensory fibres was dose dependent (Figure 1) and there was no shift in the dose-response curve when comparing normal with arthritic joints. It was not possible to determine a maximum response, as the amount that could be administered was limited by the solubility of anandamide. Figure 2 shows a typical response to anandamide (860 nmol) recorded from normal and arthritic knee joints. In normal joints the response to this dose was rapid in onset (4.5 ± 0.5 s) and of short duration (4.8 ± 1.0 s) and caused an average change in the discharge frequency of 7.2 ± 2.3 impulses s^{-1} ($n=15$ units from five animals). A similar response was obtained from arthritic joints with a response latency of 5.2 ± 0.4 s, response duration of 3.9 ± 1.1 s and a change in the discharge frequency of 9.7 ± 3.0 impulses s^{-1} ($n=11$ units from six animals). Figure 3 shows there was no significant difference in the responses when comparing normal and arthritic joints ($P > 0.05$, Mann–Whitney).

Other algogens that are capable of activating polymodal nociceptors were also examined to ascertain whether or not they were affected by the administration of anandamide (860–2900 nmol). In normal joints the response to bradykinin (9 nmol) was 1.7 ± 0.7 impulses s^{-1} and after anandamide it was not significantly different ($P > 0.05$, Mann–Whitney) with a response of 1.3 ± 0.6 impulses s^{-1} ($n=8$ units from two animals). The response to ATP (2000 nmol) was unchanged after the administration of anandamide ($P > 0.05$, Mann–Whitney) with a response of 14.8 ± 6.0 impulses s^{-1} prior to anandamide and 11.2 ± 4.7 impulses s^{-1} following ($n=5$ units from two animals). In arthritic joints after anandamide, there was no significant difference ($P > 0.05$, Mann–Whitney) in the response to bradykinin (9 nmol) or ATP (2000 nmol) with responses of 2.2 ± 0.6 impulses s^{-1} and 1.3 ± 0.5 impulses s^{-1} ($n=6$ units from three animals) before and after bradykinin respectively and 2.3 ± 1.5 impulses s^{-1} and 6.5 ± 2.5 impulses s^{-1} ($n=4$ units from three animals) before and after ATP respectively.

The response to capsaicin (9 nmol) was also examined before and after anandamide. In normal joints, the afferent discharge was 18.2 ± 5.4 impulses s^{-1} before and 8.1 ± 2.8

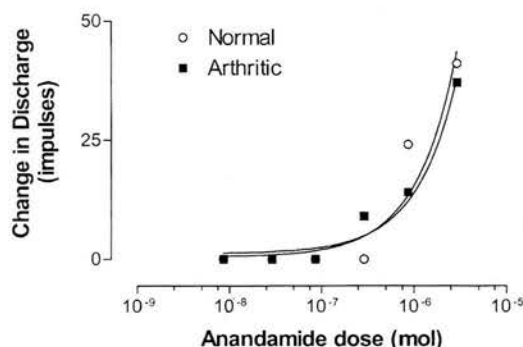


Figure 1 Dose-related increase in absolute discharge (impulses) evoked by close arterial injection of anandamide in a single C-fibre polymodal nociceptor innervating both a normal and an arthritic knee joint.

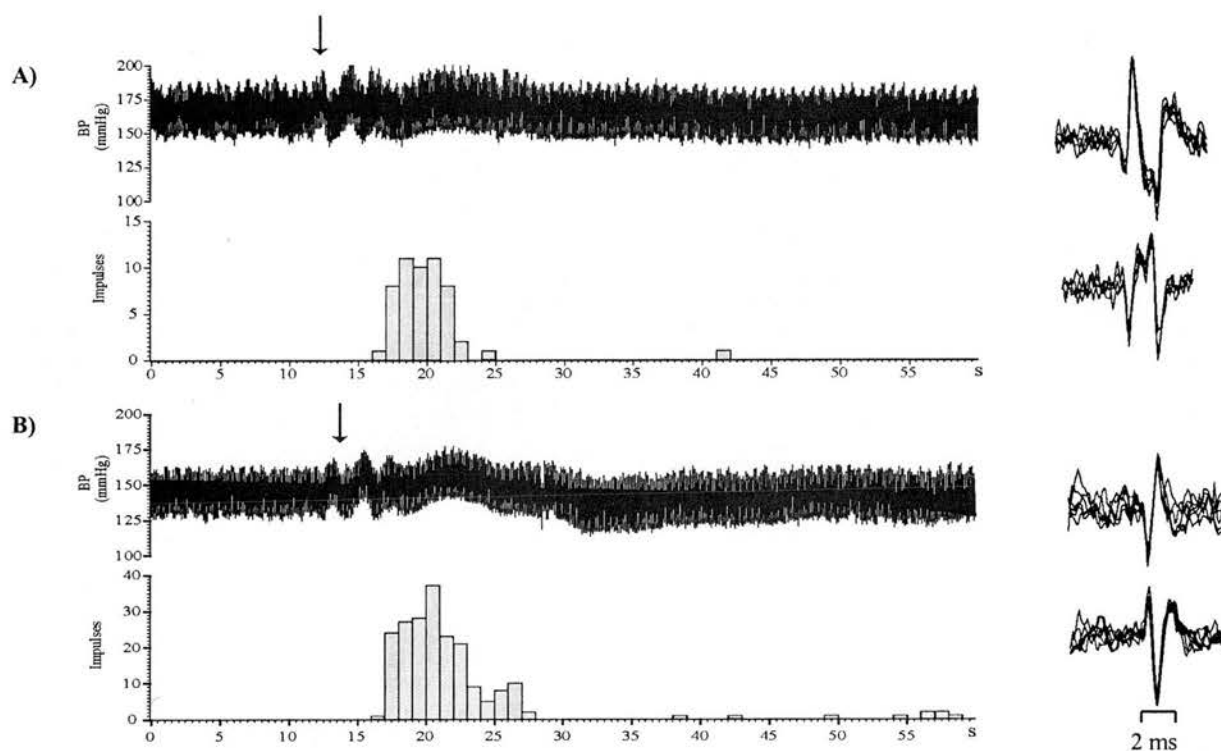


Figure 2 Spike 2 traces showing the evoked discharge from an afferent fibre of the medial articular nerve innervating the (A) normal and (B) arthritic rat knee joint evoked by close arterial injection of anandamide (860 nmol; arrow). The upper panel shows the blood pressure and the lower panel is a bar graph representing the pooled discharge of the individual units recorded from the afferent fibre. Pooled data are shown because the responses evoked are similar in all units. Discharge is displayed in 1 s bins. The panels on the right show an overlay of the individual units (2) present in the pooled discharge.

impulses s^{-1} after anandamide and this difference was not found to be significant ($P > 0.05$, Wilcoxon, $n = 12$ from four animals). Similarly, no difference was seen in the arthritic joints ($P > 0.05$, Wilcoxon) with responses of 11.6 ± 2.0 impulses s^{-1} before and 8.1 ± 2.2 impulses s^{-1} after anandamide ($n = 11$ from six animals).

Response to anandamide is abolished by VR1 antagonist capsazepine In order to confirm the algogenic effects of anandamide were attributable to activation of VR₁ receptors the effects of both capsaicin (9 nmol) and anandamide (2900 nmol) were examined in the presence of the VR₁ antagonist capsazepine (1 mg kg^{-1}). In a normal rat knee joint, capsazepine completely abolished the afferent response to both capsaicin and anandamide (Figure 4). The effect was short lasting and the response to both drugs recovered after 20 min. Furthermore the effect was specific to capsaicin and anandamide, as responses to both bradykinin (9 nmol) and $\alpha\beta$ meATP (60 nmol) were not altered by capsazepine (data not shown).

Discussion The primary finding from this *in vivo* study is that anandamide causes a rapid short-lasting excitation in a sub-population of peripheral, polymodal nociceptors in normal and arthritic rat knee joints in a dose dependent manner, producing a rapid, short-lasting excitation.

Anandamide is an agonist at CB₁ and CB₂ receptors; both these receptors are present in the periphery, with CB₁

receptors localized to the sensory nerves and the CB₂ receptors expressed on immune cells such as B-cells and natural killer cells. The ability of anandamide to activate nociceptors seems inconsistent with previously reported anti-nociceptive actions of the CB₁ receptor. Cannabinoids act on the CB₁ receptor both centrally, in the brain and spinal cord, and peripherally in the dorsal root ganglion to produce their analgesia (reviewed in Walker *et al.*, 1999 and Fuentes *et al.*, 1999). However, our studies focus in particular on the role of anandamide acting directly on peripheral nerve terminals located in an articular joint and its ability to initiate a nociceptive response. Analgesic effects of the CB₁ receptor appear to result from relatively low doses of cannabinoids, and the high doses of anandamide required to activate peripheral nociceptors in our study are likely to result from actions on a different pharmacological receptor.

The rapid onset of the response to anandamide eliminates a number of potential indirect mechanisms, such as activation of the CB₂ receptor on immune cells triggering the release of algogenic mediators. Furthermore, even allowing for the rapid metabolism of anandamide to arachadonic acid and subsequent eicosanoids, the latency of onset of the response is too short to be the result of a metabolite. The rapid response is not characteristic of G-protein coupled receptor activation, rather it is more representative of the response of a ligand gated ion-channel such as the VR₁ receptor channel (Szallasi & Blumberg, 1999).

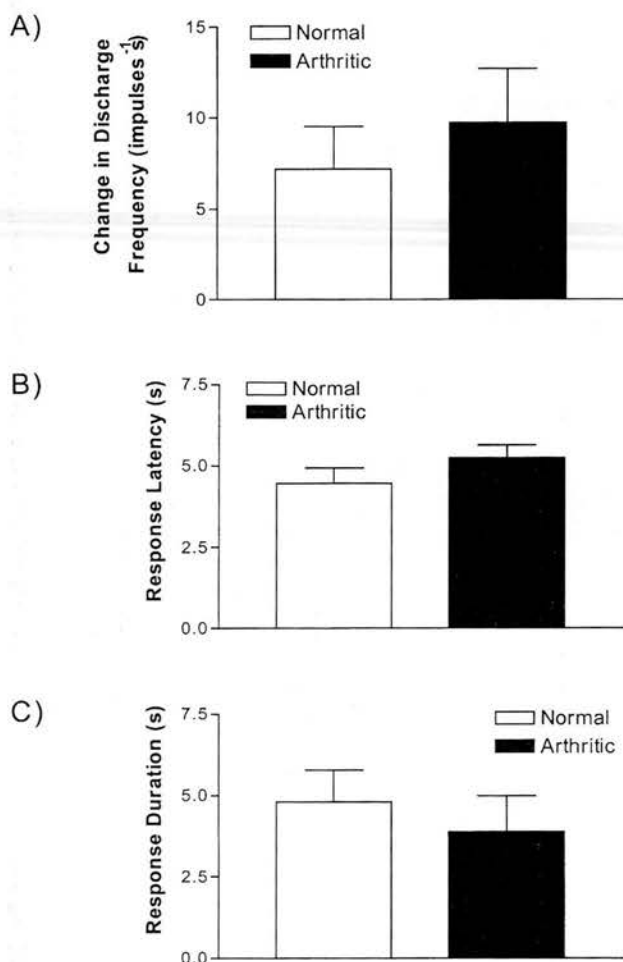


Figure 3 Comparison of the (A) increase in nociceptive discharge, (B) latency to onset, (C) duration of the response following a close arterial injection of anandamide (860 nmol). Data shown is for normal (15 units from five animals) and chronically arthritic ($n=11$ units from six animals) joints. There is no significant difference in any facet of the response between normal and arthritic joints ($P>0.05$, Mann-Whitney).

Anandamide is a full agonist at the human VR₁ receptor when expressed in HEK293 cells (Smart *et al.*, 2000), and the vasodilatory action of anandamide is a result of VR₁ mediated release of CGRP (Zygmunt *et al.*, 1999). The afferents activated by anandamide were all sensitive to capsaicin, however the corollary was untrue with 64 and 72% of capsaicin sensitive afferents being activated by anandamide in normal arthritic joints respectively. The VR₁ receptor is expressed on approximately 88% of small to medium neurones in the dorsal root ganglion (Michael & Priestley, 1999). These are the cell bodies for thinly myelinated (A δ) or unmyelinated (C) fibres running from the periphery. Although the VR₁ receptor is not present on all sensory afferents our study did not demonstrate any afferents that were sensitive to anandamide without showing sensitivity to capsaicin. Furthermore, we have shown that direct activation of capsaicin sensitive sensory nerves *in vivo* by anandamide can be abolished by the VR₁ antagonist capsazepine (1 mg kg⁻¹). Therefore, the activation of periph-

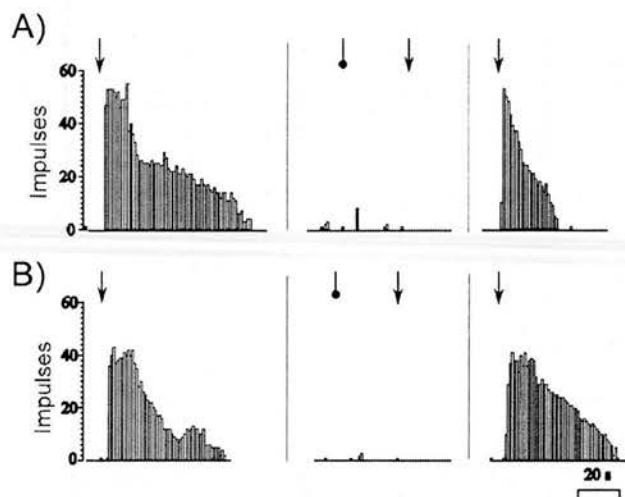


Figure 4 Afferent discharge from a single C-fibre evoked by (A) capsaicin (9 nmol) on (B) anandamide (2900 nmol) innervating a normal knee joint before and after the bolus injection of capsazepine 1 mg kg⁻¹. Capsaicin is administered at the arrows and capsazepine at the circle. The time interval between panels is 20 min.

eral nociceptors by high doses of anandamide appears to involve a VR₁-dependent mechanism. In addition, it can be concluded that if cannabinoid receptors are present on peripheral terminals they are not excitatory.

Capsaicin, even in small doses, causes desensitization of the VR₁ receptor in our preparation. However, anandamide at the doses tested did not produce desensitization either to capsaicin or itself. Further experiments are required to examine the afferent response to anandamide after a desensitizing dose of capsaicin (>86 nmol).

As stated earlier, low doses of cannabinoids can produce anti-hyperalgesia and anti-nociception when acting both centrally and peripherally. We examined the effect of the highest dose of anandamide on responses evoked by other algogens commonly used to characterize primary nociceptive afferents. Responses to three algogens (capsaicin, bradykinin, and ATP) were not significantly different after anandamide (860, 2900 nmol). Therefore anandamide did not reduce the chemosensitivity of primary sensory afferents. More detailed experiments are required to determine whether the high threshold mechanosensitivity or thermosensitivity of these afferents is affected by high doses of anandamide.

There has been recent debate in the literature that the concentrations of anandamide required to activate VR₁ are not physiologically relevant (Smart & Jerman, 2000; Szolcsanyi, 2000a,b; Zygmunt *et al.*, 2000). It is still not clear at what concentration anandamide might be present at peripheral nerve terminals, and it is possible that levels of locally produced ligand are sufficient for activation of nearby sensory nerve terminals. Similarly, it may be that there is an unknown endogenous compound similar to anandamide in structure with a higher affinity for the VR₁ receptor. Regardless of whether the ability of anandamide to activate the vanilloid receptor has any physiological significance, it does have pharmacological relevance with regard both to the pharmacological profile of anandamide and to the development of novel vanilloid receptor ligands.

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